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CHLAMYDIA TRACHOMATIS:

MOLECULAR CHARACTERISTICS AND EPIDEMIOLOGY IN FINLAND

Suvi Korhonen

ACADEMIC DISSERTATION

To be presented for public discussion with the permission of
the Faculty of Medicine of the University of Helsinki,
in Metsätalo, Lecture Hall 2, Unioninkatu 40, Helsinki,
on the 17th of April, 2020 at 12 o'clock.

Helsinki 2020

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ISBN 978-951-51-5970-0 (paperback)
ISBN 978-951-51-5971-7 (PDF)

Unigrafia Oy
Helsinki 2020

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To my family

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ABSTRACT

Chlamydia trachomatis is a common cause of sexually transmitted infections (STI). Most infections remain asymptomatic and repeated infections are common, which can lead to pelvic inflammatory disease (PID) and serious reproductive complications. *C. trachomatis* can be classified into different genotypes based on the major outer membrane protein gene (*ompA*) encoding the major outer membrane protein (MOMP): genotypes D–K cause urogenital infections and genotypes L1–L3 cause lymphogranuloma venereum (LGV).

Changes in the epidemiology of *C. trachomatis* have had a direct impact on diagnostics. LGV infection outbreaks were reported in 2003 in Europe after decades among men who have sex with men (MSM). A new variant of *C. trachomatis* (nvCT) was identified in Sweden in 2006 with a large deletion in the plasmid leading to failing detection by two nucleic acid amplification tests (NAAT). Another variant (FI-nvCT) emerged in Finland in 2019 with a nucleotide substitution in the 23 ribosomal ribonucleic acid (rRNA) likewise evading detection. Other microbes causing infections in the genitourinary tract, such as the human papillomavirus (HPV), herpes simplex virus (HSV), *Mycoplasma genitalium* and human herpesvirus 6 (HHV-6) may also contribute to the epidemiology of *C. trachomatis*. *C. trachomatis* promotes its survival inside the host cell through secreted effector proteins. The transcriptional expression of the genes encoding these effectors has mainly been studied using *C. trachomatis* reference strains propagated in cultured cells and not with currently circulating strains.

In this thesis, the *C. trachomatis ompA* genotype distribution in Finland was studied with *ompA* real-time polymerase chain reaction (PCR), and the occurrence of the Swedish nvCT was investigated with nvCT real-time PCR in urogenital specimens. The prevalence of the LGV types in extragenital specimens in Finland was investigated with *C. trachomatis* polymorphic membrane protein H (*pmpH*) gene real-time PCR and the LGV types were further typed with *ompA* real-time PCR and sequencing. The prevalence of HSV, HHV-6, HPV and *M. genitalium* in urogenital samples of *C. trachomatis* NAAT positive and negative young women in Finland was analysed with real-time PCR. Low-passage-number *C. trachomatis* clinical isolates originating from cervical swabs and reference strains were used to study the expression of *C. trachomatis ompA*, chlamydial protease-like activity factor (*cpaf*), translocated actin-recruiting phosphoprotein (*tarp*) and chlamydial cytotoxin (*tox*) genes in a cervical epithelial cell line during the chlamydial developmental cycle with real-time reverse transcriptase PCR (RT-PCR).

The three most prevalent *C. trachomatis* genotypes in Finland were E, F, and G comprising over 70% of all the genotypes identified in the urogenital samples. The percentage of infections due to genotypes F and G increased slightly in 1987–2008, but otherwise the proportion of genotypes has remained quite stable in Finland during the last ten years. Overall, the proportion of genotypes in Finland is fairly similar compared to the other countries in Europe. The Swedish nvCT was rare in Finland, as only two specimens (0.4%) containing the variant plasmid were detected. Indeed, the nvCT has seldom been reported in countries outside Sweden.

A genotyping method for identifying LGV types was set up for a diagnostic laboratory using NAATs for the detection of *C. trachomatis*. Among the *C. trachomatis* positive extragenital samples, nine samples (8%) contained LGV types and one sample (1%) contained both non-LGV and LGV types. The LGV types were detected mainly in rectal swabs and were mostly of genotype L2b. Altogether nine LGV DNA positive patients were identified and they were all MSM, and all except one were human immunodeficiency virus (HIV) positive. The data show that LGV infections among MSM also occur in Finland, which should be taken into account when considering the management of rectal *C. trachomatis* infections.

The prevalence of HPV DNA was significantly higher among the *C. trachomatis* positives than the negatives (66% vs. 25%). The prevalence of HSV (1.9% vs. 0%), HHV-6 (11% vs. 14%), and *M. genitalium* DNA (4.5% vs. 1.9%) was not significantly different between the *C. trachomatis* positive and negative women. The prevalence of HSV, HHV-6, HPV, *M. genitalium*, and the *C. trachomatis ompA* genotypes did not significantly differ between those who cleared the *C. trachomatis* infection and those who did not. The test-of-cure (TOC) samples were often (13%) positive for *C. trachomatis*. The high prevalence of HPV DNA among the *C. trachomatis* positives suggests greater sexual activity and increased risk for sexually transmitted pathogens. The overall prevalence rates of HSV, HHV-6, HPV and *M. genitalium* were similar to those reported elsewhere.

Dissimilarities were observed in the gene expression patterns between the low-passage-number *C. trachomatis* clinical isolates and the reference strains. The expression of *ompA* and the peak of *tox* expression were observed earlier in the reference strains than in most of the clinical isolates. The expression of *cpaf* was high in the reference strains compared to the clinical isolates at the mid-phase of the developmental cycle. All the strains had a similar *tarp* expression profile. Most clinical isolates showed slower growth kinetics compared to the reference strains. The use of low-passage-number *C. trachomatis* clinical isolates instead of reference strains in the gene expression studies might better reflect the situation during human infection.

TIIVISTELMÄ

Chlamydia trachomatis on yleinen sukupuoliteitse tarttuvia infektioita aiheuttava bakteeri. Suurin osa infektioista on oireettomia ja toistuvat infektiot ovat yleisiä, mikä voi johtaa sisäsynnytintulehdukseen ja vakaviin lisääntymisterveyden komplikaatioihin. *C. trachomatis* voidaan luokitella ulkomembraaniproteiinia (MOMP) koodaavan *ompA* -geenin perusteella eri genotyyppeihin: genotyypit D–K aiheuttavat urogenitaalialueen infektioita ja genotyypit L1–L3 aiheuttavat lymphogranuloma venereumia (LGV).

C. trachomatis -bakteerin epidemiologiassa on tapahtunut muutoksia, joilla on ollut suoria vaikutuksia diagnostiikkaan. Vuonna 2003 Euroopassa havaittiin vuosikymmenten jälkeen LGV-infektioita miehillä, joilla oli ollut seksiä miesten kanssa. Ruotsissa tunnistettiin vuonna 2006 uusi *C. trachomatis* -variantti (nvCT), jonka plasmidissa oleva deleetio johti sen tunnistamisen epäonnistumiseen kahdella nukleiinihapon monistustestillä. Vastaavasti Suomessa todettiin vuonna 2019 *C. trachomatis* -variantti (FI-nvCT), jonka 23S ribosomaalisessa ribonukleiinihapossa (rRNA) olevan nukleotidisubstituution takia variantti jäi löytymättä yhdellä nukleiinihapon monistustestillä. Myös muilla urogenitaalialueen infektioita aiheuttavilla mikrobeilla, kuten ihmisen papilloomaviruksella (HPV), herpes simplex -viruksella (HSV), *Mycoplasma genitalium* -bakteerilla tai ihmisen herpesvirus 6:lla (HHV-6) voi olla vaikutusta *C. trachomatis* -bakteerin epidemiologiaan. *C. trachomatis* edesauttaa selviytymistään isäntäsolun sisällä tuottamiensa efektoriproteiinien avulla. Näitä efektoriproteiineja koodaavien geenien ekspressiota on tutkittu pääasiassa soluviljelmissä kasvatettujen *C. trachomatis* -referenssikantojen avulla eikä ajankohtaisilla väestössä kiertävillä kannoilla.

Tässä väitöskirjatyössä määritettiin *C. trachomatis ompA*-genotyyppijakauma Suomessa urogenitaalialueen näytteissä reaaliaikaisella *ompA*-geeniä tunnistavalla polymeraasiketjureaktio (PCR) -testillä. Ruotsalaisen nvCT:n esiintyvyyttä urogenitaalialueen näytteissä tutkittiin reaaliaikaisella nvCT-PCR:llä. LGV-tyyppien esiintyvyyttä nielun ja peräsuolen limakalvonäytteissä Suomessa tutkittiin reaaliaikaisella *C. trachomatis* -bakteerin polymorfista membraaniproteiini H (*pmpH*) -geeniä tunnistavalla PCR:llä ja LGV-tyypit tunnistettiin tarkemmin reaaliaikaisella *ompA*-PCR:llä ja sekvensoinnilla. HSV:n, HHV-6:n, HPV:n ja *M. genitalium* -bakteerin esiintyvyyttä *C. trachomatis* -positiivisten ja -negatiivisten nuorten naisten urogenitaalialueen näytteissä Suomessa tutkittiin reaaliaikaisella PCR:llä. *C. trachomatis* -bakteerin proteaasia (*cpaf*), fosfoproteiinia (*tarp*) ja sytotoksiinia (*tox*) koodaavien geenien sekä *ompA* -geenin ekspressio bakteerin elinkierron aikana analysoitiin reaaliaikaisella käänteiskopiointi-PCR:llä (RT-PCR) kohdunkaulan epiteelisolulin-

jassa kohdunkaulan limakalvonäytteistä peräisin olevilla kliinisillä *C. trachomatis* -kannoilla ja -referenssikannoilla.

Kolme yleisintä *C. trachomatis* -genotyyppiä Suomessa olivat E, F ja G, ja niiden osuus oli yli 70% kaikista urogenitaalialueen näytteistä tunnistetuista genotyypeistä. Genotyyppien F ja G aiheuttamien infektioiden prosenttiosuus suureni hiukan vuosina 1987–2008. Viimeisen kymmenen vuoden aikana genotyyppijakauma on pysynyt melko vakaana ja se on Suomessa melko samanlainen kuin muissa Euroopan maissa. Ruotsalainen variantti oli harvinainen Suomessa, sillä vain kaksi näytettä (0,4%) sisälsi varianttiplasmidin. Tätä varianttia on löydetty harvoin Ruotsin ulkopuolelta.

Tässä työssä perustettiin diagnostisen laboratorion käyttöön soveltuva tyypitysmenetelmä LGV-tyyppien tunnistamiseksi. *C. trachomatis* -positiivisista nielun ja peräsuolen limakalvonäytteistä yhdeksän näytettä (8%) sisälsi LGV-tyypin ja yksi näyte (1%) sekä LGV- että non-LGV-tyypin. LGV-tyypit havaittiin enimmäkseen peräsuolesta otetuista näytteistä ja ne olivat pääasiassa genotyyppiä L2b. LGV-DNA-positiivisia potilaita tunnistettiin yhdeksän, joista kaikilla oli ollut miesten välistä seksiä ja kaikki paitsi yksi olivat HIV-positiivisia. Tulokset osoittavat, että LGV-infektioita esiintyy myös Suomessa lähinnä miesten välisessä seksissä, mikä tulee ottaa huomioon *C. trachomatis* -bakteerin aiheuttamien peräsuolitulehdusten diagnostiikassa ja hoidossa.

HPV-DNA:n esiintyvyys oli merkitsevästi korkeampi *C. trachomatis* -positiivisten kuin -negatiivisten joukossa (66% vs. 25%). HSV- (1,9% vs. 0%), HHV-6- (11% vs. 14%) ja *M. genitalium*-DNA:n (4,5% vs. 1,9%) esiintyvyys ei ollut merkitsevästi erilainen *C. trachomatis* -positiivisten ja -negatiivisten naisten välillä. HSV:n, HHV-6:n, HPV:n, *M. genitalium* -bakteerin ja *C. trachomatis ompA*-genotyyppien esiintyvyys ei eronnut merkittävästi niiden välillä, joiden *C. trachomatis* -infektio parani ja joiden *C. trachomatis* -infektio ei parantunut. Jälkitarkastusnäytteet olivat usein (13%) *C. trachomatis* -positiivisia. HPV-DNA:n korkea esiintyvyys *C. trachomatis* -positiivisilla viittaa suurempaan seksuaaliseen aktiivisuuteen ja lisääntyneeseen riskiin saada sukupuoliteitse tarttuva infektio. HSV:n, HHV-6:n, HPV:n ja *M. genitalium* -bakteerin esiintyvyys oli samankaltainen kuin muissa maissa raportoitu.

Kliinisten *C. trachomatis* -kantojen ja -referenssikantojen geenieksprессioprofiilien välillä havaittiin eroja. *C. trachomatis ompA*-geenin ekspressio alkoi aikaisemmin ja *tox*-geenin ekspression huippu havaittiin aikaisemmin referenssikannoilla kuin useimmilla kliinisillä kannoilla. *C. trachomatis cpaf*-geenin ekspressiotaso oli referenssikannoilla korkeampi *C. trachomatis* -bakteerin elinkierron keskivaiheessa verrattuna kliinisiin kantoihin. Kaikilla kannoilla oli samankaltainen *tarp*-geenin ekspressioprofiili. Lähes kaikkien kliinisten kantojen kasvu oli hitaampaa kuin referenssikantojen. Kliinisten *C. trachomatis* -kantojen käyttö geenieksprессiotutkimuksissa referenssikantojen sijasta voisi paremmin kuvastaa tilannetta ihmisen tartunnan aikana.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I. **Niemi* S**, Hiltunen-Back E & Puolakkainen M. *Chlamydia trachomatis* genotypes and the Swedish new variant among urogenital *Chlamydia trachomatis* strains in Finland.
Infectious Diseases in Obstetrics and Gynecology 2011; 2011:481890.
https://doi.org/10.1155/2011/481890.

II. **Korhonen S**, Hiltunen-Back E & Puolakkainen M. Genotyping of *Chlamydia trachomatis* in rectal and pharyngeal specimens: identification of LGV genotypes in Finland.
Sexually Transmitted Infections 2012; 88(6):456–9.
https://doi.org/10.1136/sextrans-2011-050458.

III. **Korhonen S**, Hokynar K, Mannonen L, Paavonen J, Hiltunen-Back E & Puolakkainen M. Transcriptional expression of the *ompA*, *cpaf*, *tarp* and *tox* genes of *Chlamydia trachomatis* clinical isolates at different stages of the developmental cycle.
Microorganisms 2019; 7(6):153.
https://doi.org/10.3390/microorganisms7060153.

IV. **Korhonen S**, Hokynar K, Eriksson T, Natunen K, Paavonen J, Lehtinen M & Puolakkainen M. The prevalence of HSV, HHV-6, HPV and *Mycoplasma genitalium* in *Chlamydia trachomatis* positive and *Chlamydia trachomatis* negative urogenital samples among young women in Finland.
Pathogens 2019; 8(4):276.
https://doi.org/10.3390/pathogens8040276.

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ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pairs
CD	Constant domain
cDNA	Complementary DNA
cpaf	Chlamydial protease-like activity factor gene
CPAF	Chlamydial protease-like activity factor
CRB	*Chlamydia*-related bacteria
DNA	Deoxyribonucleic acid
EB	Elementary body
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme immunoassay
ENA	European Nucleotide Archive
FDA	Food and Drug Administration
FI-nvCT	Finnish new variant of *Chlamydia trachomatis*
FVU	First-void urine
GE	Genome equivalent
HHV-6	Human herpesvirus 6
HIV	Human immunodeficiency virus
hpi	Hours post infection
HPV	Human papillomavirus
HSP	Heat shock protein
HSV	Herpes simplex virus
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
kb	Kilo base pairs
LPS	Lipopolysaccharide
LGV	Lymphogranuloma venereum
Mb	Mega base pairs
MIF	Micro-immunofluorescence
MLST	Multi locus sequence typing
MLVA	Multi locus variable number tandem repeat analysis
MOMP	Major outer membrane protein
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men

NAAT	Nucleic acid amplification test
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor-κB
NIDR	The National Infectious Disease Register
nvCT	New variant of *Chlamydia trachomatis*
ompA	Major outer membrane protein gene
ORF	Open reading frame
PCR	Polymerase chain reaction
Pgp	Plasmid glycoprotein
PID	Pelvic inflammatory disease
pmp	Polymorphic membrane protein gene
Pmp	Polymorphic membrane protein
POCT	Point-of-care test
PRR	Pattern recognition receptor
RB	Reticulate body
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
SDA	Strand displacement amplification
SNP	Single nucleotide polymorphism
STI	Sexually transmitted infection
T3SS	Type III secretion system
tarp	Translocated actin-recruiting phosphoprotein gene
Tarp	Translocated actin-recruiting phosphoprotein
TFI	Tubal factor infertility
TMA	Transcription mediated amplification
TOC	Test-of-cure
tox	Chlamydial cytotoxin gene
VD	Variable domain
WGS	Whole genome sequencing

1. INTRODUCTION

The global health burden due to sexually transmitted infections (STI) is enormous (Collaborators 2016). It is estimated that one million infections involving the four curable sexually transmitted diseases chlamydia, gonorrhoea, syphilis and trichomoniasis are acquired each day (Rowley et al. 2019). *Chlamydia trachomatis* is the most common bacterial cause of STIs, and it affects especially young adults (Rowley et al. 2019). Most *C. trachomatis* infections are asymptomatic and repeated infections are common, which can lead to serious sequelae of infections such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal factor infertility (TFI) (Wiesenfeld 2017).

C. trachomatis can be classified into different genotypes based on the major outer membrane protein gene (*ompA*) encoding the major outer membrane protein (MOMP) (Morré et al. 1998): genotypes D–K cause urogenital infections and genotypes L1–L3 cause lymphogranuloma venereum (LGV). LGV is an invasive STI resulting in severe inguinal lymphadenopathy (Mabey & Peeling 2002). It was previously encountered mainly in Africa, Asia and South America, but in 2003 several outbreaks of infection caused by *C. trachomatis* LGV types were observed among men who have sex with men (MSM) in the Western countries (Martin-Iguacel et al. 2010). The disease presented different clinical symptoms to those previously described, as a mild or asymptomatic proctitis was mainly observed among the patients. These LGV positive patients were also affected by high rates of other STIs, such as the human immunodeficiency virus (HIV).

Antibiotic resistance among *C. trachomatis* strains is extremely rare (Sandoz & Rockey 2010), and these pathogens have utilised other strategies for spreading. In 2006, the Swedish new variant of *C. trachomatis* (nvCT) emerged harbouring a large deletion in the chlamydial cryptic plasmid (Ripa & Nilsson 2006). This deletion site resided in the target sequences of two major nucleic acid amplification test (NAAT) manufacturers, which led to failing detection of several thousand *C. trachomatis* cases in Sweden (Jurstrand et al. 2013).

C. trachomatis co-infections with other sexually transmitted microbes are extremely common, but the interaction and consequences of these co-infections have only been partially explored. *C. trachomatis* is a known co-factor in the development of cervical cancer together with the human papillomavirus (HPV) (Castellsagué et al. 2014; Lehtinen et al. 2011). In addition, herpesviruses have been shown to interact with *C. trachomatis* by promoting persistence of *C. trachomatis* (Deka et al. 2006; Prusty et al. 2012). The data on the local and global prevalence of sexually transmitted microbes are important in planning public health interventions.

Transcriptional gene expression analysis has revealed new aspects of the biology of *C. trachomatis* infections (Belland et al. 2003; Nicholson et al. 2003). However, these studies have often used reference strains, which might be adapted to growing in cultured cell lines used in laboratories. Molecular characterization of *C. trachomatis* using low-passage-number isolates might better represent the biological properties of the strains currently causing infections. As gene expression studies are usually performed *in vitro* using cultured cell lines, the gene expression of *C. trachomatis* during human genital infection *in vivo* remains an understudied area in *Chlamydia* research.

This thesis studies the Finnish *C. trachomatis ompA* genotype distribution in urogenital samples and investigates whether the genotypes L1–L3 causing LGV also occur in Finland by examining extragenital samples. It also studies whether the Swedish nvCT has spread to Finland, and explores the prevalence of other sexually transmitted pathogens including herpes simplex virus (HSV), human herpesvirus 6 (HHV-6), HPV and *Mycoplasma genitalium* in urogenital specimens. In addition, selected low-passage-number *C. trachomatis* clinical isolates from cervical samples are characterised for transcriptional expression of genes encoding important chlamydial effector proteins.

2. REVIEW OF THE LITERATURE

2.1 CHLAMYDIALES

Chlamydia are small (0.2–1.5 μm) obligate intracellular Gram-negative bacteria of the family *Chlamydiaceae*, order *Chlamydiales*, phylum *Chlamydiae*, with a unique two-phase developmental cycle (Whitman 2015). *Chlamydia* was discovered in 1907 when the intracytoplasmic inclusions in conjunctival scrapings from orangutans were described using Giemsa staining (Halberstaedter & von Prowazek 1907). In 1959, the first genital isolate of *C. trachomatis* was recovered from the cervix of a woman with PID using the yolk sac culture technique (Jones et al. 1959).

The family *Chlamydiaceae* includes one genus, *Chlamydia*, which contains eleven established species: *C. abortus*, *C. avium*, *C. caviae*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. suis* and *C. trachomatis* (Table 1) (Sachse et al. 2015). The division of the family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila*, has been proposed (Everett et al. 1999), but this separation has later been proved invalid based on 16S ribosomal ribonucleic acid (rRNA) sequence and genomic similarity of the different *Chlamydia* species (Sachse et al. 2015). *C. trachomatis* and *C. pneumoniae* are the two major species that cause infections in humans. *C. pneumoniae* is a common cause of respiratory tract infection and community-acquired pneumonia (Roulis et al. 2013). A *C. pneumoniae* infection has also been suggested to be associated with several chronic conditions such as asthma and atherosclerosis. *C. psittaci* causes psittacosis in birds and can be zoonotically transmitted to humans, causing respiratory tract infection and pneumonia (Knittler et al. 2014).

Table 1. Characteristics of the eleven different species in the genus *Chlamydia*. Modified from Sachse et al. (2015).

Species	Natural host	Site of infection	Genome size (Mb)	Predicted proteins
C. abortus	sheep, goat	genital, respiratory	1.14	932
C. avium	pigeon, parrots	respiratory	1.05	940
C. caviae	guinea pig	ocular, genital	1.18	1005
C. felis	cat	ocular, respiratory	1.17	1013
C. gallinacea	chicken	respiratory	1.04	907
C. muridarum	rodents	gastrointestinal	1.08	911
C. pecorum	cattle, koala	gastrointestinal, respiratory, urogenital	1.11	988
C. pneumoniae	human, horse, koala	respiratory, cardiovascular	1.23	1113
C. psittaci	birds	respiratory	1.17	975
C. suis	swine	gastrointestinal, genital	1.08	931
C. trachomatis[a]	human	genital, ocular	1.04	894

Mb, mega base pairs; [a] *C. trachomatis* D/UW-3/CX (Stephens et al. 1998).

The order *Chlamydiales* has recently expanded to include at least seven additional families: *Clavichlamydiaceae*, *Criblamydiaceae*, *Parachlamydiaceae*, *Piscichlamydiaceae*, *Rhabdochlamydiaceae*, *Simkaniaceae* and *Waddliaceae* (Whitman 2015). These *Chlamydia*-related bacteria (CRB) share the intracellular lifestyle and developmental cycle of the *Chlamydia* species and have been isolated from a wide variety of hosts. It was estimated that the *Parachlamydiaceae* and *Chlamydiaceae* families diverged from the last common obligate intracellular ancestor more than 700 million years ago (Greub & Raoult 2003). The genomes of CRB are roughly two to three times larger than the genomes of the *Chlamydia* species (Taylor-Brown et al. 2015). It seems that the *Chlamydia* species have gone through massive genome reduction during co-evolution with their eukaryotic hosts (Stephens et al. 1998). The pathogenicity of different CRB to humans is only partially characterised. *Simkania negevensis* and *Parachlamydia* spp. have been associated with respiratory infections and *Waddlia chondrophila* with miscarriage and other adverse pregnancy outcomes (Taylor-Brown et al. 2015).

2.2 EPIDEMIOLOGY OF *CHLAMYDIA TRACHOMATIS*

The *C. trachomatis* urogenital infection is the most common sexually transmitted bacterial infection with an estimated 127 million cases occurring annually (Rowley et al. 2019). In 2017, 26 European countries reported more than 400 000 *C. trachomatis* cases to the European Centre for Disease Prevention and Control (ECDC) (ECDC 2019a). In 2016, the global prevalence estimate for *C. trachomatis* was 3.8% in women and 2.7% in men (Rowley et al. 2019). The estimated prevalence in 2016 was similar to the one reported in 2012, which shows that *C. trachomatis* infections are persistently endemic worldwide (Newman et al. 2015). In Finland, a similar prevalence of 3.5% was reported among 18-year-old women participating in a *C. trachomatis* screening trial (Lehtinen et al. 2018).

The notification rate for the 22 European countries with comprehensive surveillance systems was 146 *C. trachomatis* cases per 100 000 population in 2017 (ECDC 2019a). In Finland, the incidence of *C. trachomatis* was 270 cases per 100 000 inhabitants in 2018. In the other Nordic countries, the incidence of *C. trachomatis* was 337/100 000 in Sweden, 478/100 000 in Norway and 573/100 000 in Denmark in 2017. All the Nordic countries, including Finland, use widespread opportunistic screening. Comparing the incidence or notification rates between countries is challenging because of the differences in screening approaches, diagnostic methods, case finding and reporting.

In Finland, *C. trachomatis* infections have been notified by laboratories to the National Infectious Disease Register (NIDR), maintained by the Finnish Institute for Health and Welfare since 1995. The annual number of notified cases has been stable and has remained around 12 000–14 000 for the last ten years (Figure 1) (NIDR 2019). In 2018, there were 14 839 notified *C. trachomatis* cases in Finland. Most infections were reported among young women of Finnish origin: 58% were women, 79% were 15–29 years old and 91% were Finnish. On an average, *C. trachomatis* infections occur at a younger age in women compared to men: two-thirds of the women were infected under 25 years of age, while one-third of the men were infected under 25 years of age.

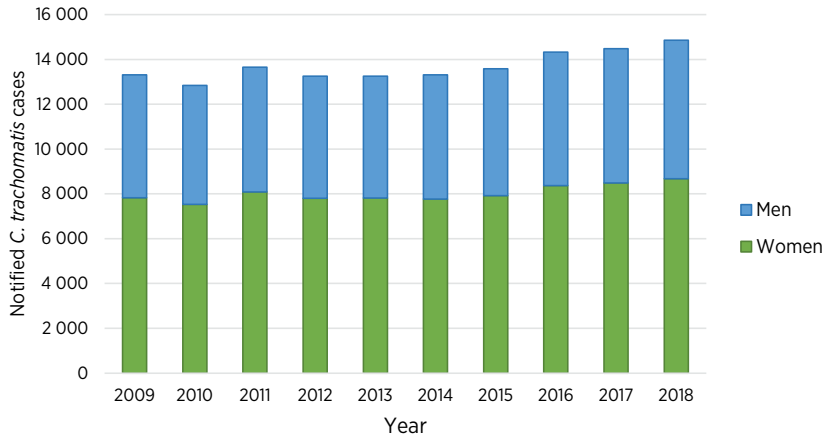

Figure 1. The number of *C. trachomatis* cases in men and women notified by laboratories in Finland in 2009–2018. (NIDR 2019).

2.3 *C. TRACHOMATIS* INFECTIONS

2.3.1 UROGENITAL INFECTION

The analysis of the MOMP of *C. trachomatis* and the *ompA* gene encoding MOMP has led to the identification of 20 different genotypes of *C. trachomatis* (Morré et al. 1998; Wang et al. 1973). Genotypes A–C cause ocular infections, genotypes D–K cause urogenital infections and genotypes L1–L3 cause LGV. In women, *C. trachomatis* infects the columnar epithelial cells of the cervix and most (70–90%) of the infections remain asymptomatic (Peipert 2003). Symptoms include abnormal vaginal discharge and dysuria (Wiesenfeld 2017). *C. trachomatis* infections can also occur at extragenital sites, such as pharynx and rectum, and these infections are often asymptomatic (Chan et al. 2016). In a large study performed in the USA, the prevalence of *C. trachomatis* was 9% in rectum and 3% in pharynx among women (Trebach et al. 2015). Untreated or repeated *C. trachomatis* infections can ascend from the cervix to the uterus and fallopian tubes and lead to PID (Brunham et al. 2015). PID is a risk factor for tubal damage which can lead to ectopic pregnancy and TFI.

Ectopic pregnancy can be a life-threatening condition and it occurs when the blastocyst implants outside the uterus in either the fallopian tubes, ovaries or abdomen (Adachi et al. 2016). Most cases of TFI are due to salpingitis, an inflammation of the epithelial surfaces of the fallopian tube leading to tubal occlusion (Tsevat et al. 2017). It has been suggested that the inflammatory responses to the chlamydial heat shock protein (HSP) might be responsible for tubal damage that

leads to reproductive complications (Adachi et al. 2016). A *C. trachomatis* infection during pregnancy can lead to miscarriage, premature rupture of membranes, stillbirth, preterm delivery and low birth weight of the infant by either direct fetal infection, placental damage or severe maternal illness (Adachi et al. 2016). The mechanisms by which a *C. trachomatis* infection can lead to adverse pregnancy outcomes are only partially known. *C. trachomatis* might directly infect the fetus, triggering a harmful inflammatory response, or the maternal inflammatory response might induce embryonic rejection due to homology of the chlamydial and human HSPs (Adachi et al. 2016).

Approximating the risk for PID, ectopic pregnancy and TFI after a *C. trachomatis* infection is challenging because of the asymptomatic nature of the infection, duration of time between diagnosis, lack of specific diagnostic criteria and differences in study design (Davies et al. 2016; Hoenderboom et al. 2019). However, several large cohort studies have shown that the risk of reproductive complications is increased with a positive *C. trachomatis* test (Davies et al. 2016; den Heijer et al. 2019; Hoenderboom et al. 2019; Reekie et al. 2018), and this risk is further increased with a repeated infection (Davies et al. 2016; den Heijer et al. 2019). A modelling study suggested that the risk for PID is 17% and the risk for salpingitis is 7% following an untreated *C. trachomatis* infection (Price et al. 2016). In addition, the same study estimated that 20% of PID, 5% of ectopic pregnancy and 29% of TFI are due to *C. trachomatis*. Although the incidence of ectopic pregnancy and TFI is low, PID caused by *C. trachomatis* remains the most important preventable cause of reproductive complications (Low et al. 2016).

C. trachomatis causes urethritis in men and approximately 30–50% of the infections are asymptomatic (Peipert 2003). When symptomatic, urethral discharge and dysuria may be reported. Extragenital infections occur also in men: the prevalence of *C. trachomatis* was 9% in rectum and 2% in pharynx among heterosexual men in a large study performed in the USA (Trebach et al. 2015). Sequelae of a *C. trachomatis* infection in men include epididymitis and epididymo-orchitis, and *C. trachomatis* might also be the causative agent of prostatitis (Mackern-Oberti et al. 2013). There is conflicting evidence whether chronic *C. trachomatis* infections leading to these conditions may be the cause of sub-fertility or infertility in men (Gimenes et al. 2014). Several studies have shown that a *C. trachomatis* infection is associated with poor sperm quality, impaired sperm fertilising capacity and DNA integrity, while other studies demonstrated no association between *C. trachomatis* infection and altered sperm quality (Gimenes et al. 2014).

A urogenital *C. trachomatis* infection can lead to reactive arthritis in both men and women which often shows a remitting-relapsing disease phenotype (Gerard et al. 2013). It is estimated that approximately 5% of individuals with a *C. trachomatis* genital infection will develop acute reactive arthritis, and approximately a further

half of these develop chronic arthritis (Kvien et al. 1994). In the synovial tissue of patients with acute or chronic arthritis, persistent forms of *C. trachomatis* can be detected (Gerard et al. 2013). In addition, *C. trachomatis* types identified in the synovia were of ocular genotypes A–C and not of genital genotypes D–K, but the reason behind this is unknown (Gerard et al. 2010).

The natural history of untreated *C. trachomatis* infections varies: infections can persist without symptoms for many months, can descend to the upper genital tract or can spontaneously resolve (Geisler et al. 2013). Human studies on the duration of an untreated, uncomplicated genital *C. trachomatis* infection have shown that approximately half of the infections spontaneously clear in one year after the positive *C. trachomatis* test (Geisler 2010). Some degree of protective immunity against *C. trachomatis* develops after a genital infection possibly providing protection against reinfection, but the duration of this immunity is unclear (Batteiger, Xu, et al. 2010). The effect of protective immunity was shown among sex workers, where resistance to a *C. trachomatis* infection correlated with duration of prostitution, regardless of age (Brunham et al. 1996). In addition, a recent study showed that young women who spontaneously cleared a *C. trachomatis* infection were able to resist reinfection (Geisler et al. 2013).

Despite the development of a partial protective immunity against *C. trachomatis*, repeated infections are extremely common. It is estimated that up to 20% of patients are reinfected within 12 months of treatment (Hosenfeld et al. 2009; Walker et al. 2012). Similar rates were reported also in Finland, where 25% of women and 20% of men acquired a repeated infection (Wikström et al. 2012). Repeated *C. trachomatis* infections result from reinfection due to sexual contact with either an untreated existing partner or a new infected partner, or from failure of antibiotic therapy. In a study performed in the USA among adolescent women, 84% of the infections were reinfections and 14% were treatment failures (Batteiger, Tu, et al. 2010).

The high rates of *C. trachomatis* infections observed worldwide might be due to early treatment interfering with the development of protective immunity, which enhances susceptibility to repeated infections (Brunham & Rekart 2008). This arrested immunity hypothesis is probably not the only explanation for the high *C. trachomatis* rates, as screening programmes, access to testing and changes in sexual behaviour can also contribute. Arrested immunity might also explain the declining rates of *C. trachomatis* sequelae observed in many countries, as early treatment prevents the infection from descending to the upper genital tract (Brunham & Rekart 2008). Supporting the hypothesis, the number of *C. trachomatis* cases notified by laboratories increased at the same time as sequelae, such as PID, preterm delivery and ectopic pregnancy, and the *C. trachomatis* seroprevalence among fertile-aged women decreased in Finland in 1983–2003 (Lyytikäinen et al. 2008). Recently, it was shown that asymptomatic gastrointestinal *C. trachomatis*

infections occur in humans and present a source of persistent infection (Rank & Yeruva 2014). The immune system and azithromycin used to treat urogenital infections might be unable to clear *C. trachomatis* from the gut, so that women cured of a genital infection could become reinfected by autoinoculation from the gastrointestinal tract, which might partly explain their high reinfection rate.

2.3.2 NEONATAL INFECTION

C. trachomatis can be vertically transmitted from mother to infant through an infected birth canal during child delivery resulting in neonatal conjunctivitis or pneumonia (Darville 2005). *C. trachomatis* may also infect the urogenital tract and rectum of the infant and these infections can be asymptomatic. Neonatal conjunctivitis or ophthalmia neonatorum usually develops 5–14 days after birth (Darville 2005). The manifestations of the disease vary from mild conjunctival infection to severe mucopurulent conjunctivitis, but healing without complications usually occurs if treated early. Neonatal *C. trachomatis* pneumonia is usually mild and afebrile and occurs between one and three months of age (Darville 2005). Symptoms of neonatal pneumonia include nasal obstruction and/or discharge, tachypnea and a repetitive staccato cough. Untreated pneumonia can persist for several weeks and can lead to poor feeding and diminished weight gain. *C. trachomatis* neonatal pneumonia may also lead to asthma and chronic lung disease later in life (Adachi et al. 2016).

Prenatal screening and treatment of *C. trachomatis* infections in pregnant women have lowered the incidence of neonatal infections, but only few countries have implemented such programmes (Adachi et al. 2016). In Finland, opportunistic screening is available to both symptomatic and asymptomatic pregnant women. The estimated risk of transmission of *C. trachomatis* to the infant during childbirth is approximately 50%, and pneumonia develops in 10–20% of infants exposed to *C. trachomatis* (Hammerschlag 2011). In Finland, *C. trachomatis* infections among infants are rare (0.22 per 1000 live births) and the risk of vertical transmission from *C. trachomatis* positive mothers to neonates is significantly lower than previously reported, less than 2% (Honkila et al. 2017).

2.3.3 LYMPHOGRANULOMA VENEREUM (LGV)

In contrast to the mucosal urogenital infections caused by *C. trachomatis* genotypes D–K, *C. trachomatis* invasive genotypes L1–L3 cause an LGV infection in the lymphatic system. Sexually transmitted LGV infections are endemic in parts of Africa, Asia, South America, and in some Caribbean islands (Mabey & Peeling 2002). The classic clinical manifestation of an LGV infection progresses in different stages (de Vries 2019). In the primary stage, a painless papule or a shallow ulcer appears in the epithelia at the infection site and it usually heals in a few days. After 2–6 weeks, in the secondary stage, the infection spreads to the inguinal or femoral lymph nodes with the formation of severe lymphadenopathy, strictures and fistulas. If left untreated, late manifestations of LGV in the tertiary stage involve severe disfiguring conditions of the external genitalia, such as elephantiasis and esthiomene.

After the introduction of antibiotics, classic LGV infections were rarely encountered in the Western countries and were considered mainly as imports from endemic countries (Mabey & Peeling 2002). In 2003, several outbreaks due to the *C. trachomatis* genotypes L were reported in Europe, North America and Australia (Martin-Iguacel et al. 2010; Nieuwenhuis et al. 2004). LGV infections were observed mainly among MSM who were often HIV positive, had other concomitant sexually transmitted infections such as syphilis, gonorrhoea or hepatitis C, and presented high risk sexual behaviour involving international networks. In contrast to the classic lymphadenopathy, LGV infections manifested mainly as proctitis with symptoms such as anal cramps, tenesmus, constipation, discharge and pain. In addition, asymptomatic proctitis was reported.

In Finland, LGV infections have been notified by clinicians to the NIDR since 1932. At the beginning of the 1930s, there were almost 200 reported cases of LGV in Finland, and the prevalence of LGV decreased towards the end of the 1930s with 29 LGV cases in 1939 (Olin 1941). Since then LGV infections remained practically non-existent in Finland until 2009.

2.3.4 TRACHOMA

Trachoma, an eye infection caused by *C. trachomatis* genotypes A–C, is the most common infectious cause of blindness worldwide (Taylor et al. 2014). Trachoma affects 21 million people with approximately 2.2 million blinded or severely visually impaired, and 7.3 million people suffering from corneal damage (Mariotti et al. 2009). These eye infections are rarely seen in Europe and North America and occur mostly in the developing countries of Africa and Asia. Infections are transmitted through infected ocular secretions among children whose facial cleanliness is poor. Repeated infections with *C. trachomatis* in childhood result in severe conjunctival inflammation, conjunctival scarring, distortion of the eyelid and inturned eyelashes

touching the cornea which can lead to blindness in later life. Control programmes against trachoma have been implemented, and trachoma has been successfully eliminated in several countries (Taylor et al. 2014). The goal of these programmes is to eliminate trachoma worldwide by 2020. Control measures include surgery of the eyelids, mass distribution of antibiotics, promotion of facial cleanliness, and environmental improvement with access to clean water and sanitation.

2.4 DEVELOPMENTAL CYCLE OF *C. TRACHOMATIS*

As an obligate intracellular bacterium, *C. trachomatis* replicates only in a host cell with a special two-phase growth cycle, which involves complex interactions between the bacteria and the host (AbdelRahman & Belland 2005) (Figure 2). The intracellular lifestyle of *C. trachomatis* enables the acquirement of essential host cell nutrients and offers protection from the defence mechanisms of the host cell.

The developmental cycle begins when the infectious form, the elementary body (EB), attaches to the host cell membrane through interactions between bacterial adhesins, host receptors and host heparan sulphate proteoglycans (Elwell et al. 2016). EBs inject the pre-synthesised effector proteins such as the translocated actin-recruiting phosphoprotein (Tarp) through the type III secretion system (T3SS) to the host cytosol (Clifton et al. 2004). These effectors modulate host cell signalling and actin cytoskeleton rearrangement and facilitate the entry of the EB into the host cell via endocytosis. The EB has previously been considered to be metabolically inactive, but recent studies show that also EBs present metabolic activity and utilise different energy sources to maintain infectivity (Grieshaber et al. 2018; Omsland et al. 2012).

After endocytosis, the EBs are enclosed in a membrane-bound vacuole called inclusion and start to transform to larger and highly metabolically active reticulate bodies (RBs) (Elwell et al. 2016). Early RB effectors produced at 2–8 hours post infection (hpi) facilitate the remodelling of the inclusion membrane, redirect exocytic vesicle to the inclusion to promote nutrient acquisition, and prevent fusion with lysosomes by inhibiting interaction with the endocytic pathway. RBs are transported along microtubules to the microtubule organising centers or centrosomes near the peri-Golgi region. At 12 hpi RBs actively replicate and divide by binary fission, and they are closely associated to the inner face of the inclusion membrane. During this phase, the size of the inclusion grows and RBs must acquire essential lipids for growth and survival (Gitsels et al. 2019). To achieve this, the inclusion interacts with intercepting vesicles from organelles in the peri-Golgi region through vesicular and non-vesicular transport pathways. In addition to the acquisition of nutrients and lipids, RB effectors activate proliferation and survival pathways, inhibit apoptotic pathways and modulate the innate immune signalling of the host (Elwell et al. 2016).

After several hours of replication and cell division, RBs asynchronously transform back to EBs at 18–24 hpi. Late *C. trachomatis* effectors include outer membrane complex proteins and DNA binding histone proteins, which condense DNA and switch off the transcription of many genes (Elwell et al. 2016). Late effector proteins are packaged into forming EBs to be released in the next cycle of infection, and EBs exit the host cell via cell lysis or extrusion at 48 hpi.

Under certain environmental pressure, RBs can transform to non-dividing persistent forms in which they are viable but non-cultivable (Panzetta et al. 2018). This persistence can be induced by beta-lactam antibiotics, interferon-gamma (IFN-γ), and by the deprivation of iron or amino acids. Persistent or aberrant forms continue to replicate DNA and synthesise proteins, but cell division is halted. Persistent forms can transform back to RBs when the environmental stress factors are removed. Although persistence might lead to chronic inflammation and scarring, it is not known whether persistence occurs *in vivo*. The evidence for persistence in humans is so far indirect, as it is based on the direct observation of persistent forms in infected tissue, such as endocervical cells (Lewis et al. 2014). Persistent forms have been shown to be resistant to azithromycin, which is of importance as azithromycin is the first-line antibiotic used to treat *C. trachomatis* urogenital infections (Wyrick & Knight 2004).

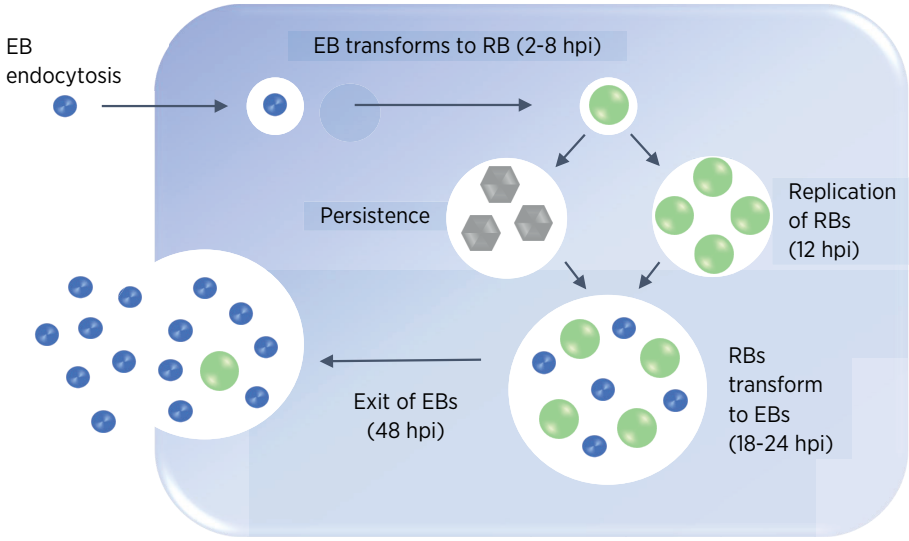

Figure 2. The developmental cycle of *C. trachomatis*. The infectious elementary body (EB) enters the host cell by endocytosis and transforms to a metabolically active reticulate body (RB) at 2–8 hours post infection (hpi). Inside the inclusion, RBs replicate and divide by binary fission (12 hpi) or they can transform to non-dividing persistent forms in the presence of growth inhibitors. Persistent forms can transform back to RBs when the inhibitor is removed. After several hours of replication, RBs begin to transform back to EBs (18–24 hpi) and EBs exit the host cell to infect new cells (48 hpi). Modified from Brunham & Rey-Ladino (2005).

2.5 HOST IMMUNE RESPONSE AGAINST *C. TRACHOMATIS*

Replication inside the inclusion offers protection to *C. trachomatis*, as exposure to antibodies and antigen-processing and -presentation mechanism of the host is limited. The immune response of the host against *C. trachomatis* is dynamic and involves cells and mediators from both innate and adaptive immunity (Vasilevsky et al. 2014). *C. trachomatis* is recognised by the host cell by receptors of the innate immune system called pattern recognition receptors (PRR), such as the toll-like receptors (Chen et al. 2019). PRRs recognise certain structures of bacteria called pathogen associated molecular patterns, for example peptidoglycan or lipopolysaccharide (LPS). This triggers the release of pro-inflammatory cytokines and chemokines, which attracts inflammatory cells. The cytokines produced by epithelial cells polarise the immune response towards a protective Type 1 helper T cell response, and immunity to *C. trachomatis* is largely mediated by $CD4^+$ T cells producing IFN-γ. The secreted chemokines and T cell responses mediating the elimination of bacteria are also responsible for *C. trachomatis* related immunopathology, such as tissue damage and scarring, and may result from a shift from Type 1 helper T cell to Type 2 helper T cell immunity (Vasilevsky et al. 2014).

C. trachomatis can manipulate the immune response of the host by several mechanisms, which can rescue the bacteria from clearance, but also contribute to the asymptomatic nature of chlamydial infections. IFN-γ secreted by T cells decreases the intracellular tryptophan levels via indoleamine 2,3-dioxygenase. *Chlamydia* are auxotrophic for tryptophan and respond to this stress with the formation of persistent, non-dividing forms, which may transform back to RBs when tryptophan is again available. Urogenital *C. trachomatis* strains can respond to this deprivation by synthesising tryptophan from indole, which is produced by other bacteria in the vaginal flora (Fehlner-Gardiner et al. 2002). Nuclear factor-κB (NF-κB) is an important protein complex of the host that regulates DNA replication, cytokine production and cell survival. *C. trachomatis* has evolved different strategies to block the transcription of NF-κB to enhance intracellular survival (Chen et al. 2019). *C. trachomatis* can block the degradation of the NF-κB retention factor and prevent the nuclear translocation of NF-κB which inhibits NF-κB transcription.

2.6 *C. TRACHOMATIS* GENOME

The sequence of the first *C. trachomatis* genome (genotype D) was published in 1998 (Stephens et al. 1998), and was followed by genomes from *C. trachomatis* genotype A (Carlson et al. 2005), and two genomes from *C. trachomatis* genotype L2 (Thomson et al. 2008). These genomes showed a strikingly high degree of similarity

in terms of size, nucleotide sequence similarity (>99% identical) and synteny (Table 2). Twenty years later whole genome sequencing (WGS) technologies have advanced in terms of reduced time and cost, and now there are approximately 180 *C. trachomatis* genomes in the National Center for Biotechnology Information (NCBI) genome database (https://www.ncbi.nlm.nih.gov/genome/), and approximately 200 genomes in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/) of the European Bioinformatics Institute.

Table 2. Properties of the *C. trachomatis* genomes of genotypes D, A and L2. Modified from Thomson et al. (2008).

Strain	**UW-3/CX[a]**	**Har-13[b]**	**434/Bu[c]**
Genotype	D	A	L2
Biovar	genital	ocular	LGV
Chromosome size (bp)	1 042 519	1 044 459	1 038 842
Plasmid size (bp)	7 493	7 510	7 499
G+C content (%)	41.27	41.27	41.33
Predicted proteins	894	920	889

LGV, lymphogranuloma venereum; bp, base pairs;
[a] (Stephens et al. 1998); [b] (Carlson et al. 2005); [c] (Thomson et al. 2008)

WGS of *C. trachomatis* revealed a relatively small genome of 1.04 mega base pair (Mb) (Carlson et al. 2005; Stephens et al. 1998; Thomson et al. 2008). The *C. trachomatis* genome encodes a set of genes close to the minimum needed for DNA replication, transcription and translation. However, DNA repair and recombination mechanisms are vastly represented in the genome. The *C. trachomatis* genome includes the genes needed for the essential functions in aerobic respiration, but lacks many genes encoding metabolic enzymes, which makes this bacterium reliant on the host for many of its metabolic requirements. *C. trachomatis* was initially considered to be a strict energy parasite, but genome analysis revealed the necessary genes for adenosine triphosphate (ATP) generation and ATP translocases employed to acquire ATP from the host. Some *C. trachomatis* genes were acquired from eukaryotes, such as the genes encoding chromatin-associated domain proteins, which may act in chlamydial nucleoid condensation and de-condensation. The *C. trachomatis* genome includes the genes for peptidoglycan biosynthesis, and *Chlamydia* are susceptible to antibiotics that target peptidoglycan, but it was just until recently that the *C. trachomatis* cell wall was finally proven to contain peptidoglycan (Liechti et al. 2014). The importance of pathogenicity is well represented in the *C. trachomatis*

genome as approximately 10% of the genome encodes virulence effectors (Elwell et al. 2016).

In addition to the similarities between the genomes of genotypes A, D and L2, differences exist especially in the 50 kilo base pair (kb) plasticity zone, in the *ompA* gene encoding the MOMP, among the polymorphic membrane protein gene family members (*pmp*) encoding polymorphic membrane proteins (Pmp), and among *inc* genes encoding inclusion membrane proteins. The chlamydial cytotoxin gene (*tox*) residing in the plasticity zone is intact in the genital *C. trachomatis* strains, but in ocular and LGV strains there are extensive deletions in the *tox* gene (Belland et al. 2001; Carlson et al. 2004). Genital genotypes of *C. trachomatis* have a *trpBA* operon encoding tryptophan synthase, but ocular genotypes have accumulated mutations in the genes of the operon that inactivate the enzyme (Fehlner-Gardiner et al. 2002). Sequence variation within *pmp* genes distinguishes between the ocular, genital and LGV types of *C. trachomatis* (Stothard et al. 2003). These dissimilarities in the genomes might explain the differences in tissue tropism and disease outcome among the ocular, genital and LGV types of *C. trachomatis*.

The *C. trachomatis* genome was considered to be highly conserved due to the obligate intracellular niche of *Chlamydia*. However, WGS analyses have revealed that gene duplications, point mutations, insertions, deletions and horizontal gene transfer occur in the chlamydial genome (Abdelsamed et al. 2013). In addition, it is now known that in *Chlamydia* recombination frequently takes place both *in vitro* (Jeffrey et al. 2013) and *in vivo* (Hadfield et al. 2017).

Many *Chlamydia* species including *C. trachomatis* contain a highly conserved 7.5 kb long cryptic plasmid with copy numbers of 4–10 per host cell (Zhong 2017). Plasmid-free strains have been described which suggests that the cryptic plasmid is not essential for growth or infectivity of the bacteria. However, the plasmid is an important virulence factor of *C. trachomatis*. Plasmid-free *C. trachomatis* causes attenuated infections with decreased loads of organisms or shorter duration that spontaneously resolve with reduced or no post-infection pathology. The plasmid encodes eight open reading frames (ORFs) called plasmid glycoproteins (Pgp) 1–8. Putative functions of several ORFs have been assigned based on the homology to known proteins in the public databases. Pgp1 is homologous to a helicase involved in the unwinding of double-stranded DNA during replication. Pgp7 and Pgp8 are involved in the plasmid replication, and Pgp5 is a homologue of plasmid-partitioning proteins. Pgp2 and Pgp6 are *Chlamydia*-specific proteins showing little or no homology to proteins in the public databases. Pgp3 is highly conserved among *Chlamydia* and is secreted into the host cytosol of infected cells, and it may neutralise host antimicrobial peptides. Pgp4 regulates the expression of Pgp3 and is largely responsible for the plasmid-mediated regulation of chlamydial chromosomal gene expression (Song et al. 2013). The most highly regulated genes include those

encoding for glycogen synthase, Pmp-like proteins and putative T3SS effectors. The plasmid is also a negative regulator of the expression of the chlamydial protease-like activity factor (CPAF) for the L2 genotype (Patton et al. 2018).

Two factors have limited research on the biology of *Chlamydia* infections. The first is the lack of a host-free system for growing *Chlamydia* and the second is the absence of methods for transformation and genetic manipulation of *Chlamydia*. Unfortunately, a host-free method for *C. trachomatis* culture is still lacking, but recently there have been major advances in the techniques addressing the latter issue. A shuttle vector based on the chlamydial plasmid was stably introduced into *C. trachomatis* (Wang et al. 2011). This transformation technique was widely adopted by other groups with minor variations. Also genetic manipulation of *Chlamydia* is now possible using a method that allows targeting chlamydial genes for deletion or allelic exchange, as well as curing plasmids with a novel suicide vector (Mueller et al. 2016). Recently, a clustered regularly-interspaced short palindromic repeats interference was used to repress the gene expression of *C. trachomatis* (Ouellette 2018).

2.7 *C. TRACHOMATIS* GENE EXPRESSION

C. trachomatis gene expression exhibits temporal patterns, which regulate the different stages of the chlamydial developmental cycle. Early genes are expressed within one to three hours after endocytosis, mid-cycle genes start expression during RB replication and cell division, and late genes are transcribed when RBs convert to EBs (Belland et al. 2003; Nicholson et al. 2003; Shaw et al. 2000).

The temporal gene expression of *C. trachomatis in vitro* has been studied with Northern blot analysis (Stephens, Wagar, & Edman 1988) and real-time polymerase chain reaction (PCR) (Mathews et al. 1999), or reverse transcriptase-PCR (RT-PCR) using agarose gel electrophoresis (Shaw et al. 2000). Whole transcriptome analyses have been performed with single isolates of *C. pneumoniae* (Albrecht et al. 2011; Maurer et al. 2007) and *C. trachomatis* (Albrecht et al. 2010; Ferreira et al. 2017; Humphrys et al. 2013) at a specific phase of the developmental cycle.

The *C. trachomatis* gene expression studies have mainly been performed with laboratory reference strains propagated in the laboratory for decades (Belland et al. 2003; Nicholson et al. 2003). Previous studies have demonstrated the effects of long-term propagation on the *C. trachomatis* genome, gene expression and growth dynamics. In one study, repeated laboratory passages resulted in interrupting mutations in the gene CT135 among low-passage-number genital clinical strains (Bonner et al. 2015). In another study, the gene expression of many virulence factors including the Tarp was downregulated, and an increase in growth rates

was observed among clinical strains (ocular, genital and LGV) after long-term propagation (Borges et al. 2015). In addition, heterogeneity in the population of the genital strains affecting the sequence of the *tox* gene led to a potentially disrupted cytotoxin. Only a single study observed few adaptive mutations and no significant differences in the growth rates in a genital clinical strain after one year of serial passaging (Borges et al. 2013).

The studies analysing the gene expression of both *C. trachomatis* reference and low-passage-number clinical strains during the chlamydial developmental cycle are few. One study analysed the expression of the *C. trachomatis ompA* gene throughout the developmental cycle using both reference and clinical strains, and in this study the gene expression levels of the reference and clinical strains did not correlate (Nunes et al. 2007). To our knowledge, no studies have been conducted analysing the expression of chlamydial virulence genes, such as *tarp* and *tox*, or *cpaf* which encodes a chlamydial protease, using clinical low-passage-number strains during the different stages of the developmental cycle.

Gene expression of *C. trachomatis* in cell culture is fairly well known, but gene expression during *in vivo* infection remains somewhat unexplored. In addition, it is not known to what extent gene expression *in vitro* correlates to the situation in human infection. Only a few studies are describing the *in vivo* expression of selected *C. trachomatis* genes. The expression of chlamydial HSP genes was studied directly from cervical cells (Jha et al. 2009) and synovial biopsies (Gerard et al. 2013).

2.8 *C. TRACHOMATIS* PROTEOME AND EFFECTOR PROTEINS

The genome of *C. trachomatis* encodes approximately 900 predicted proteins (Carlson et al. 2005; Stephens et al. 1998; Thomson et al. 2008). About two-thirds of the proteins are shared across species, which reflects the genetic conservation of *Chlamydia* (Elwell et al. 2016). Effector proteins are translocated to the bacterial surface by the type V secretion system, to the inclusion lumen by the type II secretion system, and into the host cell or inclusion membrane by the T3SS. *C. trachomatis* has been shown to deliver at least 60 different effector proteins to the inclusion membrane and the host cytoplasm (Bugalhão & Mota 2019). A quantitative proteomics study on the two different developmental forms of *C. trachomatis* revealed that EBs are primed for high T3SS capacity and for generating a burst of energy via glucose catabolism to fuel the EB to RB transformation (Saka et al. 2011). RBs are primed for robust protein synthesis, nutrient transport, the accumulation of ATP, efficient replication, and ultimately for the transformation of RBs to EBs. Another proteomics study showed that EBs are metabolically active and have the capacity to generate ATP, and most of the enzymes involved in peptidoglycan

biosynthesis were detected in RBs (Skipp et al. 2016). The data achieved with the proteomic analyses correlate well with the results from genomic (Carlson et al. 2005; Stephens et al. 1998; Thomson et al. 2008) and transcriptional studies (Albrecht et al. 2010; Belland et al. 2003).

Among the most extensively studied *C. trachomatis* effector proteins are CPAF (coding sequence number CT858), Tarp (CT456) and chlamydial cytotoxin (CT166). CPAF is a potent type II secreted protease which is detected in the host cell cytosol (Snavely et al. 2014; Zhong et al. 2001). Many of the previously reported effects of CPAF have been questioned (Chen et al. 2012), but it seems that CPAF has a role in the later stages of infection during the exit of EBs (Snavely et al. 2014). Tarp is translocated into the host cell by T3SS where it is tyrosine phosphorylated by host kinases (Clifton et al. 2004). It is a multidomain protein that directly associates with actin and is involved in several different signalling events that lead to actin recruitment and internalisation of the EB promoting intracellular survival of *Chlamydia* (Elwell et al. 2016). The chlamydial cytotoxin residing in the plasticity zone inactivates intracellular regulatory molecules such as the GTP-binding proteins of the Rho/Ras family by glycosylation, which induces actin depolymerisation of the host cell promoting the entry of the EB (Belland et al. 2001; Thalmann et al. 2010). Cytotoxin has also been shown to be involved in delayed cell cycle progression and multinucleation of the host cell (Bothe et al. 2015).

MOMP is the principal outer membrane protein and surface antigen of *C. trachomatis*. The size of MOMP is 40 kDa and it constitutes 60% of the proteins on the outer membrane (Caldwell et al. 1981). MOMP maintains the structure of EB, and inside the host cell MOMP transforms into a porin through which RBs can acquire nutrients. This transformation occurs by sulphur bridges within MOMP and between MOMP and other outer membrane proteins. MOMP consists of five constant domains (CD) CD1–5 and four variable domains (VD) VD1–4 (Stephens et al. 1987) (Figure 3). The hydrophobic membrane-spanning CDs anchor MOMP to the outer membrane. The hydrophilic, surface-exposed epitopes of VD1, VD2 and VD4 stimulate the production of neutralising antibodies. In addition, the epitopes of VD1 and VD2 stimulate the production of serotype-specific antibodies, and the epitopes of VD4 stimulate the production of serogroup and *C. trachomatis* specific antibodies (Baehr et al. 1988; Stephens, Wagar, & Schoolnik 1988).

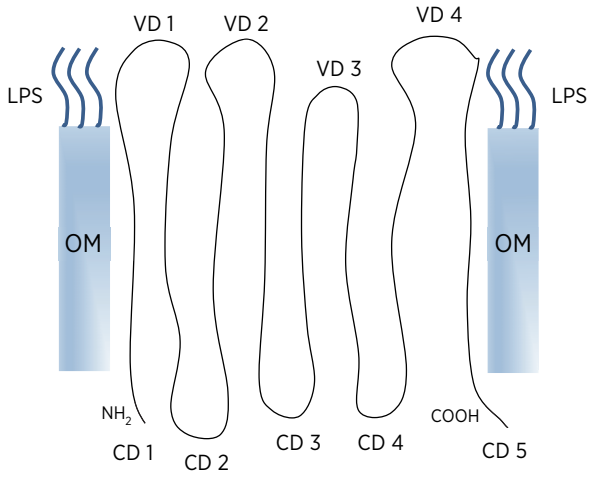

Figure 3. The structure of the major outer membrane protein (MOMP) of *C. trachomatis*. MOMP is anchored to the lipopolysaccharide (LPS)-containing outer membrane (OM) by five hydrophobic constant domains (CD) CD1–5. The four surface-exposed, hydrophilic variable domains (VD) VD1–4 stimulate the production of antibodies. Modified from Kim & DeMars (2001).

Another group of proteins located on the outer membrane of *C. trachomatis* are the Pmps. Genes *pmpA–I* encode nine different Pmps, which are autotransporters and adhesins, important in the initial phase of a *C. trachomatis* infection (Vasilevsky et al. 2016). Pmps as typical autotransporters include a cleavable N-terminal Sec-dependent leader sequence for localisation to the membrane, a passenger domain for surface localisation or secretion, and a C-terminal β-barrel translocator sequence for outer membrane translocation. Pmps are large (90–187 kDa) (Stothard et al. 2003), and like MOMP, Pmps as surface-exposed proteins elicit a host immune response, of which PmpB, PmpC, PmpD and PmpI are the most immunogenic (Vasilevsky et al. 2016). Pmps, as well as MOMP, are potential candidates utilised as components of a subunit *C. trachomatis* vaccine (de la Maza et al. 2017).

2.9 TYPING OF *C. TRACHOMATIS*

Typing of *C. trachomatis* is used for epidemiological, diagnostic and research purposes. It can reveal transmission networks, distinguish reinfection from treatment failure, and separate LGV from non-LGV infections (de Vries et al. 2015). First *C. trachomatis* typing methods were based on the characterization of the MOMP using specific antibodies. Serotyping was first performed with polyclonal and later with monoclonal antibodies using the micro-immunofluorescence (MIF) test (Wang et al. 1985). This method requires high quantities of living bacteria, non-standardised

antibodies, and it is expensive and laborious. The sero-immunological analysis of the *C. trachomatis* MOMP led to the identification of 15 different serotypes (Wang et al. 1973). These serotypes are divided into three different groups: the B group includes serotypes B, Ba, E, D, L1 and L2, the C group includes serotypes C, A, H, I, J, K and L3, and the intermediate group includes serotypes F and G. Sequence differences in the *ompA* gene encoding MOMP, especially in the regions encoding the variable domains of MOMP, later confirmed this discrimination (Morré et al. 1998). The serological properties and the *ompA* gene sequences within a group are almost identical (Morré et al. 1998; Yuan et al. 1989).

Molecular typing methods followed serotyping, and the culture of *C. trachomatis* was no longer necessary. The first genotyping methods were mainly based on the analysis of the *ompA* gene sequences encoding the VD1–4 of MOMP. With the restriction fragment length polymorphism (RFLP) method, the *C. trachomatis ompA* gene is amplified with PCR, PCR products are digested with different restriction enzymes, restriction products are analysed on agarose gel electrophoresis and the results are compared to type strains (Frost et al. 1991). The limiting factor with the RFLP method is the number of different restriction enzymes available. The *ompA* gene can also be amplified by PCR and sequenced (Bandea et al. 2001), or the different genotypes can be detected with specific fluorescent probes by real-time PCR (Jalal et al. 2007). Contrary to traditional serotyping which does not reveal mutations, *ompA* genotyping may do so.

More detailed typing compared to the serotyping or *ompA* genotyping methods was achieved with high-resolution typing techniques, such as multi locus sequence typing (MLST) and multi locus variable number tandem repeat analysis (MLVA). MLST is used to analyse sequences of several polymorphic loci: each locus is assigned an allele number, and allele numbers constitute the profile of the strain i.e. the sequence type. MLST methods developed for *C. trachomatis* have analysed highly variable regions (Klint et al. 2007) and house-keeping genes (Pannekoek et al. 2008). In MLVA, several genomic variable number of tandem repeats loci are analysed. The number of repeats in different loci is determined, and the number series describes the profile of the strain and designates the sequence type (Pedersen et al. 2008). The genetic loci used in these MLST and MLVA techniques have proven to be very stable after multiple rounds of cell culture, which makes these methods suitable for use in the epidemiologic analyses of *C. trachomatis* (Labiran et al. 2012).

WGS can produce a wealth of data, which can be used in discriminating even the most closely related *C. trachomatis* strains. In the future, the focus of genome analysis will most probably be switched from sequencing single genomes to performing large-scale whole population analyses focusing on fine-scale sequence analysis searching for single nucleotide polymorphisms (SNP) in different *C. trachomatis* strains (Hadfield et al. 2017). Previously, samples had to be cultured

to produce large quantities of bacterial genomes for WGS, but now culture-independent methods are also available (Seth-Smith et al. 2013).

2.10 DIAGNOSIS OF *C. TRACHOMATIS* INFECTIONS

2.10.1 CULTURE

C. trachomatis can be isolated using cell lines such as McCoy, HeLa229 and Buffalo Green Monkey Kidney cells (Papp et al. 2014). Specimens are centrifuged onto confluent cell monolayers and incubated with growth medium including cycloheximide for 48–72 hours (Ripa & Mårdh 1977). Infected cells develop intracytoplasmic inclusions which are detected by staining with fluorescein-conjugated antibodies against chlamydial antigens such as MOMP or LPS. Until the introduction of NAATs, cell culture was the most sensitive method for detecting *C. trachomatis*, but now cell culture is usually used as a diagnostic test for *C. trachomatis* only when NAATs are not available or affordable. Cell culture is very specific, but the sensitivity is approximately 60–80% compared to the commercially available NAATs (Meyer 2016). In addition, cell culture is non-standardised, technically complex, labour intensive and expensive, and the specimen collection and transport are critical. However, the capacity to culture *C. trachomatis* should be maintained at least in some centralised laboratories to support surveillance and research, for example when new variants emerge.

2.10.2 NUCLEIC ACID AMPLIFICATION TESTS

At present NAATs are recommended for the laboratory diagnosis of *C. trachomatis* infections (Lanjouw et al. 2016; Papp et al. 2014). These tests are extremely specific and sensitive enough to detect small quantities of chlamydial nucleic acid. As these tests do not detect viable organisms, transport and storage conditions are not critical. The use of first-void urine (FVU), and urethral, cervical and vaginal swabs have been validated by the manufacturers of NAATs. Extragenital specimens, such as pharyngeal and rectal swabs, are cleared by the Food and Drug Administration (FDA) for two commercially available *C. trachomatis* NAATs (Aptima Combo 2 Assay, Hologic, Marlborough, MA, USA and Xpert Ct/NG, Cepheid, Sunnyvale, CA, USA). For several other NAATs sufficient evidence supports the use of these tests in the detection of *C. trachomatis* at extragenital sites (Lanjouw et al. 2016; Papp et al. 2014). In addition, home-based self-collection for *C. trachomatis* testing has been accepted with comparable sensitivity and specificity to clinician-collected specimens

(Fajardo-Bernal et al. 2015). In Finland, home-based sampling is provided by some laboratories in the public and private sector. The self-obtained FVU and vaginal swab samples are sent via mail to the laboratory, and the results are sent to the patient by a short message service.

Five NAAT assay platforms are FDA-cleared for the detection of *C. trachomatis*: Abbott RealTime CT/NG (Abbott Molecular, Des Plaines, IL, USA), Aptima Combo 2/Aptima CT assays, BD ProbeTec assay (Becton Dickinson, Eysins, Switzerland), Cobas CT/NG test (Roche Diagnostics GmbH, Mannheim, Germany), and Xpert CT/NG assay (Papp et al. 2014) (Table 3). These technologies amplify chlamydial plasmid DNA, chromosomal DNA, or rRNA with methods based on PCR, transcription mediated amplification (TMA), or strand displacement amplification (SDA). The Abbott and Roche NAATs also have dual targets for *C. trachomatis*. Targeting the chlamydial plasmid or rRNA results in increased sensitivity of the test, as these targets are present in multiple copies in the bacterium compared to the single copy of the chlamydial genome (Chernesky et al. 2014). In addition, these NAATs are often combined with the detection of another STI pathogen, *Neisseria gonorrhoeae*.

Table 3. The nucleic acid targets and amplification methods of the five major manufacturers of *C. trachomatis* NAATs.

NAAT	Target	Method	Manufacturer
Abbott RealTime CT/NG	plasmid DNA (two targets)	PCR	Abbott Molecular
Aptima Combo 2 assay	23S rRNA	TMA	Hologic
Aptima CT assay	16S rRNA	TMA	Hologic
BD ProbeTec ET CT/GC assay	plasmid DNA	SDA	Becton Dickinson
Cobas CT/NG test	chromosomal and plasmid DNA	PCR	Roche
Xpert CT/NG assay	chromosomal DNA	PCR	Cepheid

NAAT, nucleic acid amplification test; PCR, polymerase chain reaction; TMA, transcription mediated amplification; SDA, strand displacement amplification.

The commercial NAATs used to detect *C. trachomatis* are highly sensitive and specific but do not differentiate between genotypes. For detecting the *C. trachomatis* LGV types, there are commercial genital ulcer multiplex NAATs available, but these tests are not evaluated in the context of rectal LGV infections (de Vries et al. 2019). LGV genotypes are mainly identified using in-house PCR tests targeting either the *ompA* or *pmpH* genes in centralised laboratories (Morré et al. 2008).

2.10.3 SEROLOGY

In the European guideline or in the recommendation by Centers for Disease Control and Prevention, USA, serology is not recommended for the diagnosis of acute uncomplicated *C. trachomatis* infections, because anti-chlamydial antibodies are detectable several weeks after initial infection or may not develop at all (Lanjouw et al. 2016; Papp et al. 2014). In addition, serological tests are not as sensitive and specific as NAATs and cannot differentiate between past and current infection. However, serology might have some value in the diagnosis of ascending infections and reactive arthritis, and in infertility work-up (Lanjouw et al. 2016). In these cases, *C. trachomatis* can be undetectable in the urogenital specimens and serological data may be used to evaluate the role of *C. trachomatis* in the infection. Serological assays can represent an alternative method for the diagnosis for LGV, especially when LGV specific tests are not available (de Vries et al. 2019). A high antibody titre, especially anti-MOMP immunoglobulin (Ig) A antibodies, in a patient with symptoms suggestive of an LGV infection supports the diagnosis. However, a low titre does not exclude LGV, nor does a high titre in a patient without LGV

symptomology confirm LGV infection. Chlamydial antibodies can be detected with complement fixation and MIF tests. In addition, enzyme immunoassays (EIA), immunoblots or line assays are currently used in *C. trachomatis* serology (Meyer 2016). Novel biomarkers for more specific serology are being developed (Rantsi et al. 2019).

2.10.4 POINT-OF-CARE TESTS

C. trachomatis NAATs are usually performed in large, centralised laboratories which requires transportation of samples and reporting the test results back to the clinicians. Therefore, with NAAT-based diagnostics, the patients need to come back to the clinic for the results. This can lead to delayed treatment or no treatment at all if the patients do not return and further transmit the infection. This has led to the development of point-of-care tests (POCT) (Kelly et al. 2017). The currently available, mostly immunochromatographic POCTs are based on lateral flow technology and detect *Chlamydia* LPS antigen in genital swabs or urine. Unfortunately, these tests have poor sensitivity compared to NAATs and therefore are not suitable to be used as a screening test for *C. trachomatis* infection (van Dommelen et al. 2010). Other tests based on antigen detection are also available, such as EIAs and direct fluorescent antibody tests, but NAATs are approximately 20–35% more sensitive than these tests and their use in routine diagnostics is not recommended (Papp et al. 2014).

The Cepheid Xpert CT/NG assay was the first commercial rapid NAAT that provided point-of-care testing for *C. trachomatis* and *N. gonorrhoeae* in 90 minutes (Gaydos et al. 2013). New, more rapid tests based on nucleic acid detection are currently being developed as not all patients are willing to wait for 90 minutes for the results. These rapid, sensitive and affordable POCTs are desperately needed, especially in low-resource and remote areas (Kelly et al. 2017).

2.10.5 TESTING GUIDELINES

The recommended specimens for the diagnosis of urogenital *C. trachomatis* infections with NAATs are FVU in men and a self- or clinician-collected vaginal swab in women (Lanjouw et al. 2016; Papp et al. 2014). In men, a urethral swab sample is equivalent to a urine sample in detecting *C. trachomatis*, but clearly more invasive. In women, cervical samples taken during a pelvic examination are also appropriate, but vaginal swabs have proved to be as sensitive as cervical samples in NAATs. Due to decreased sensitivity, FVU is not the recommended specimen type for women. Annual *C. trachomatis* testing is recommended for all sexually active

young women and men under 25 years of age, and repeated testing in 3–6 months should be offered to those young individuals who test positive for *C. trachomatis* (Lanjouw et al. 2016; Workowski & Bolan 2015). International guidelines do not encourage a test-of-cure (TOC) to be routinely performed unless adherence in therapy is questioned, symptoms persist, or reinfection is suspected. However, the Finnish national treatment guideline recommends a TOC four weeks after the treatment (Collaborators 2018). This is the only difference between the Finnish and the international guidelines. Routine annual screening of extragenital sites including rectum and pharynx is recommended for MSM (Workowski & Bolan 2015), but such recommendations are not available for women or heterosexual men, although extragenital infections among these individuals are not uncommon (Chan et al. 2016).

A current European guideline on the management of LGV recommends that LGV should be tested in all MSM with a *C. trachomatis* positive anorectal sample, contacts of confirmed LGV index cases, patients with symptoms suspected of having LGV, HIV-positive MSM and MSM using anti-HIV medication (de Vries et al. 2019). Other *C. trachomatis* positive sites (urethra, urine or pharynx) can be tested for LGV if symptoms persist despite antibiotic treatment. A two-test procedure is recommended: first a commercial NAAT for detecting *C. trachomatis* and a second in-house NAAT for detecting LGV types (de Vries et al. 2019; Papp et al. 2014). However, the sensitivities of the various in-house NAATs are generally lower compared to the sensitivities of the commercial NAATs. LGV routine molecular diagnostics should be available in all countries and it can be centralised at a national reference laboratory.

2.10.6 VARIANTS EVADING DETECTION

In southwest Sweden, Halland county, an unexpected fall of 25% in *C. trachomatis* cases was observed in 2005–2006, even though the number of patients tested had remained similar compared to the year before (Ripa & Nilsson 2006). In Sweden, commercial NAATs from Abbott and Roche were used in the detection of *C. trachomatis*, which at that time detected the cryptic plasmid of *C. trachomatis*. A subset of samples (n=1700) was analysed with another commercial NAAT Artus (ARTUS, Hamburg, Germany) that targets the *C. trachomatis ompA* gene, and 13% of the samples were found positive with the Artus test only. Selected strains were sequenced and the nvCT was found with a 377 base pair (bp) deletion in the ORF1 of the plasmid (Ripa & Nilsson 2007). All the strains were of genotype E. The deletion involved the entire target area in the Abbott test and a major part of the target area in the Roche test. In the Swedish counties where tests from Abbott

and Roche were used, the nvCT comprised 20–65% of the *C. trachomatis* cases (Herrmann 2007). In the counties that used the Becton Dickinson test, with the target area residing outside the deletion in the plasmid, the nvCT comprised 7–20% of the *C. trachomatis* cases (Herrmann 2008). Retrospective analysis showed that the nvCT was present already in 2003, and it has been estimated that there were 15 000 missed *C. trachomatis* cases caused by the failing detection of the nvCT (Jurstrand et al. 2013). Until 2008, it was not known if the nvCT had also spread to Finland.

Another *C. trachomatis* variant was identified in Finland in February 2019. A discrepancy in results obtained with two different NAATs used to detect *C. trachomatis* was observed in the Clinical Microbiology Laboratory of Turku University Hospital (Rantakokko-Jalava et al. 2019). An FVU sample from a patient was positive for *C. trachomatis* with the Allplex STI Essential test (Seegene, Seoul, Korea), and a urethral swab specimen taken on the same day was negative for *C. trachomatis* with the Aptima Combo 2 test which targets chlamydial 23S rRNA. Later the same samples were confirmed to be positive for *C. trachomatis* with the Aptima CT test, which detects 16S rRNA. Sequencing of the selected strains revealed a nucleotide substitution in the 23 rRNA gene in position 1515 (C to T) which is not present in any of the *C. trachomatis* reference strain sequences deposited in the GenBank. The strains were all of genotype E. It was estimated that in 2019 more than 200 *C. trachomatis* cases were missed due to the Finnish new variant of *C. trachomatis* (FI-nvCT) generating false negative/equivocal Aptima Combo 2 results (Hokynar et al. 2019). However, no decrease in the number of *C. trachomatis* cases notified by laboratories was observed in Finland in 2011–2019. Outside Finland, two cases of FI-nvCT were detected in Örebro county, Sweden (Unemo et al. 2019), and 81 *C. trachomatis* cases due to the FI-nvCT were identified in Norway (Johansen et al. 2019). Just when the FI-nvCT emerged is uncertain: the earliest available specimen that contained the FI-nvCT was taken in Turku in June 2018 (Hokynar et al. 2019).

2.11 TREATMENT OF *C. TRACHOMATIS* INFECTIONS

The international and Finnish national treatment guidelines recommend doxycycline 100 mg twice a day orally for seven days or azithromycin 1 g single oral dose in the treatment of uncomplicated urogenital *C. trachomatis* infections and *C. trachomatis* conjunctivitis (Collaborators 2018; Lanjouw et al. 2016; Workowski & Bolan 2015). The cure rates with these two regimens are similar and exceed 95% (Geisler et al. 2015). For uncomplicated non-LGV rectal and pharyngeal infections, the recommendation is the same as for urogenital infections, although doxycycline is

preferred to azithromycin in treating rectal infections. If azithromycin is used to treat rectal infections, a TOC should be taken. Higher cure rates of rectal *C. trachomatis* infections have been observed with doxycycline compared to azithromycin (Kong et al. 2015).

The recommended treatment for both symptomatic and asymptomatic LGV infections is doxycycline 100 mg twice a day orally for 21 days suggested by international and Finnish national guidelines (Collaborators 2018; de Vries et al. 2019; Workowski & Bolan 2015). Alternative antibiotic therapy is erythromycin 500 mg four times a day orally for 21 days. If specific tests for LGV are not available, patients suspected of having LGV should be treated empirically with doxycycline for three weeks.

Chlamydia can develop resistance to antibiotics through point mutations *in vitro* and these mutations can be passed on to other strains through horizontal gene transfer and homologous recombination (Sandoz & Rockey 2010). However, there is no evidence of any stable genetic or phenotypic antimicrobial resistance in *C. trachomatis* hampering treatment in clinical settings (Hadfield et al. 2017; Sandoz & Rockey 2010). A few clinical treatment failures with azithromycin 1 g single oral dose have been described (Geisler et al. 2015), but they are more likely explained by reinfection, poor compliance or tolerance to treatment, or detection of non-viable *C. trachomatis* with NAATs from a TOC sample taken too early, than by the existence of antimicrobial resistance.

2.12. CONTROL OF *C. TRACHOMATIS* INFECTIONS

2.12.1 SCREENING

The goal of the *C. trachomatis* infection control is to prevent the development of adverse sequelae such as PID, ectopic pregnancy and TFI by identifying and treating the infected individuals. Control strategies against STIs such as *C. trachomatis* infections include screening with highly sensitive and specific detection methods, antibiotic treatment of patients according to treatment guidelines, efficient contact tracing, notification and treatment, health promotion advice, and follow-up of patients (Lanjouw et al. 2016).

The effect of screening in the reduction of prevalence and incidence of *C. trachomatis* infections and reproductive complications has been analysed, and the results have been contradictory. A systematic review and meta-analysis evaluated randomised controlled trials of *C. trachomatis* screening and concluded that the effect of screening was low in reducing the prevalence and incidence of *C. trachomatis* infections (Low et al. 2016). This observation was confirmed in

a recent cluster-randomised controlled trial performed in Australia (Hocking et al. 2018). However, a large-scale population testing was successful in reducing *C. trachomatis* prevalence and declining the average duration of infection in England, although this effect was later partly reversed (Lewis & White 2018). Similarly in Finland, a population-based *C. trachomatis* screening trial resulted in a significant reduction of prevalence from 3.5% to 2.3% among young women (Lehtinen et al. 2018). *C. trachomatis* screening efforts may have at least a moderate effect in preventing *C. trachomatis* associated sequelae, mainly PID (Low et al. 2016). Indeed, the declining rates in reproductive complications have been witnessed in several countries (Lyytikäinen et al. 2008; Rekart et al. 2013). This data on the effect of screening in the reduction of prevalence and incidence of *C. trachomatis* infections and sequelae have prompted to enhance the management of *C. trachomatis* infections and disease with efforts in efficient patient control, counselling and education, alongside with *C. trachomatis* screening (Unemo et al. 2017).

The surveillance of *C. trachomatis* infections is an important part of the *C. trachomatis* infection control which should be executed both at the national and international levels. In Finland, *C. trachomatis* infections are notified by laboratories and LGV infections are notified by clinicians to the NIDR maintained by the Finnish Institute for Health and Welfare according to the Communicable Diseases Act. NIDR is a publicly open database available on the internet. In Europe the ECDC, and globally the World Health Organization contribute to the surveillance of communicable diseases including *C. trachomatis* and publish reports on the incidence and prevalence of *C. trachomatis* infections. In addition, monitoring the rates of clinically diagnosed PID, ectopic pregnancy and TFI is important in assessing the effects of the national *C. trachomatis* infection control measures.

2.12.2 VACCINE

As the screening and treatment of *C. trachomatis* infections might not result in a significant reduction in incidence and prevalence, other control strategies are needed. Ultimately, the development of a *C. trachomatis* vaccine is needed for the permanent decline of prevalence rates. A successful vaccine must trigger the T cell-mediated immune response in the genital mucosa. *C. trachomatis* vaccine development has been challenged by the poor understanding of the immune response in the female genital tract, the lack of adjuvants that target vaccines to the genital mucosa, the poor knowledge of the specific antigens that induce a protective immune response, and the lack of tools to genetically manipulate *C. trachomatis* (Brunham & Rey-Ladino 2005). The first human vaccine trials involved the administration of

whole inactivated EBs which led to partial short-lived protection but aggravated the symptoms of reinfections in some individuals (Grayston & Wang 1978). Thereafter, the research focus has been in the development of recombinant subunit vaccines that are based on individual *C. trachomatis* protein antigens, and the selection of a suitable adjuvant and delivery system.

Despite the research efforts, licensed *C. trachomatis* vaccines are not available. Recently, a genital *Chlamydia* vaccine candidate entered phase one human clinical trial for the first time (Abraham et al. 2019). The recombinant vaccine includes antigen CTH522 which is composed of segments of the MOMP from different genotypes adjuvanted with either CAF01 liposomes or aluminium hydroxide. These vaccines, administered both intramuscularly and intranasally, were safe and tolerated, and induced anti-CTH522 IgG seroconversion and cell-mediated immune responses in the participants. This holds promise for the development of an efficient *C. trachomatis* vaccine.

2.13. OTHER SEXUALLY TRANSMITTED MICROBES

A *C. trachomatis* co-infection with other sexually transmitted pathogens is common (Rowley et al. 2019). STIs including *C. trachomatis* increase the risk of HIV acquisition and transmission (Galvin & Cohen 2004). Increased susceptibility to HIV might be due to mucosal disruption and the recruitment of leucocytes, especially the target cells of HIV $CD4^+$ T cells to the endocervix. *C. trachomatis* also increases HIV shedding in the genital tract.

Sexually transmitted genital herpes caused by an HSV1 or HSV2 infection is extremely common with 544 million cases annually (Looker, Magaret, May, et al. 2015; Looker, Magaret, Turner, et al. 2015). The global prevalence estimate for a genital HSV infection was 15% (18% in women and 12% in men) in 2012. After the initial infection, HSV establishes lifelong latency during which the viral gene expression is limited and no symptoms occur (Jaishankar & Shukla 2016). Upon reactivation infectious viral particles and viral DNA are shed, and clinical disease can recur producing genital ulcers.

HHV-6 might also be sexually transmitted as it is shed in the genital tract of women (Leach et al. 1994). HHV-6 now includes two different viruses, HHV-6A and HHV-6B, with different epidemiological and biological characteristics and disease associations (Ablashi et al. 2014). Although less is known about the epidemiology of HHV-6A, HHV-6B is a ubiquitous virus. HHV-6B infection usually occurs early in childhood and can cause fever and rash (exanthema subitum) (Agut et al. 2015). HHV-6 infections including primary infections and reactivation can be asymptomatic. The seroprevalence of HHV-6 among adults is approximately 98%

(Ward et al. 1993). HHV-6 can integrate into the host telomeres, and less than 1% of individuals have HHV-6 DNA in their chromosomes (Agut et al. 2015). In addition to the possible sexual transmission, HHV-6 is known to be vertically transmitted from mother to infant and through saliva (Agut et al. 2015).

Genital HPV infections are extremely common, and the disease burden associated with HPV is enormous with 12% worldwide prevalence in women with normal cervical cytology (Forman et al. 2012). Many HPV infections clear spontaneously, but especially infections caused by the high-risk HPV genotypes can lead to cervical cancer, one of the leading cancers in women worldwide (Joura et al. 2014). Sero-epidemiologic and PCR-based studies suggest that *C. trachomatis*, together with HPV, increases the risk of development of cervical dysplasia, which can progress to cervical cancer (Castellsagué et al. 2014; Lehtinen et al. 2011). The exact mechanisms of infection on the development of precancerous lesions are not known, but *C. trachomatis* possesses several mechanisms to modulate the host cell signalling pathways, induce DNA damage, inhibit apoptosis and induce chronic inflammation (Gagnaire et al. 2017). The HPV vaccination programmes implemented in several high-income countries have succeeded in declining the infectious load of HPV and preventing HPV-related outcomes (Gottlieb & Johnston 2017).

M. genitalium is an emerging sexually transmitted pathogen, which is strongly associated with non-gonococcal and non-chlamydial urethritis in men (Taylor-Robinson & Jensen 2011). In women, *M. genitalium* can cause urethritis, cervicitis, endometritis, and PID. The increasing macrolide and fluoroquinolone resistance of *M. genitalium* is problematic since these two groups of antibiotics are virtually the only options to treat *M. genitalium* infections (Unemo & Jensen 2017). Resistance to macrolides is due to SNPs in the 23S rRNA gene of *M.* genitalium, and mutations in the DNA gyrase and topoisomerase IV genes leading to amino acid changes confer resistance to fluoroquinolones in *M. genitalium*. *N. gonorrhoeae* is the second most common bacterium causing sexually transmitted infections, with an estimated 87 million gonorrhoea cases occurring annually (Rowley et al. 2019). Like *M. genitalium*, *N. gonorrhoeae* has developed resistance against virtually all antimicrobials used in the treatment of gonorrhoea (Unemo & Jensen 2017). The widespread use of the macrolide azithromycin in the treatment of *C. trachomatis* infections has most probably contributed to the resistance in *M. genitalium* and *N. gonorrhoeae* (Unemo & Jensen 2017).

In Finland, *C. trachomatis* and *N. gonorrhoeae* infections are reported to the NIDR. HSV, HPV and *M. genitalium* infections are not notifiable diseases in Finland, and the prevalence rates of infections caused by these microbes are not known. In addition, the prevalence of HHV-6 in the genital tract has not been reported in Finland.

3. AIMS OF THE STUDY

This study aimed to determine the *ompA* genotype distribution of *C. trachomatis*, and the prevalence of *C. trachomatis* LGV types, the Swedish nvCT, and urogenital HSV, HHV-6, HPV and *M. genitalium* in Finland. Another aim was to study the transcriptional expression of the selected *C. trachomatis* genes during *in vitro* and *in vivo* infections.

The detailed aims of the individual studies were:

i. To determine the *ompA* genotype distribution of *C. trachomatis* and to investigate the occurrence of the Swedish nvCT among urogenital samples in Finland.

ii. To set up an LGV genotyping method suitable for a diagnostic laboratory using NAATs in the detection of *C. trachomatis*, to investigate the prevalence of *C. trachomatis* LGV types and to determine the *ompA* genotype distribution of *C. trachomatis* among rectal and pharyngeal samples in Finland.

iii. To study the expression of the *C. trachomatis ompA*, *cpaf*, *tarp* and *tox* genes during *in vitro* infection in a cervical epithelial cell line, using low-passage-number clinical isolates. Another aim was to evaluate the expression of the same genes *in vivo* in cervical swab specimens collected from patients with a *C. trachomatis* infection.

iv. To determine the prevalence rates of HSV, HHV-6, HPV and *M. genitalium*, and mutations leading to macrolide resistance in *M. genitalium* in *C. trachomatis* positive and *C. trachomatis* negative urogenital samples among young women in Finland. Another aim was to study whether co-infections or the *C. trachomatis* genotype affect the clearance of *C. trachomatis*.

4. MATERIALS AND METHODS

4.1 CLINICAL SAMPLES (I, II, III, IV)

The material for the *C. trachomatis ompA* genotyping included 160 unselected *C. trachomatis* NAAT positive samples from women and men sent for *C. trachomatis*, or *C. trachomatis* and *N. gonorrhoeae* testing at HUSLAB, Helsinki, Finland in 2008 (I). The 160 samples included FVUs (n=82), cervical/vaginal swabs (n=75) and conjunctival swabs (n=3). The samples were collected with the Aptima Urine Specimen Collection Kit for Male and Female Urine Specimens or with the Aptima Unisex Specimen Collection Kit for Endocervical and Male Urethral Swab Specimens, and were tested with the Aptima Combo 2 Assay (Hologic, Marlborough, MA, USA) (n=84). The samples collected with the Amplicor CT/NG Specimen Preparation Kit were tested with the Cobas TaqMan CT Test (Roche Diagnostics GmbH, Mannheim, Germany) (n=76).

The occurrence of the Swedish nvCT was analysed in 495 urogenital samples from women and men sent for *C. trachomatis*, or *C. trachomatis* and *N. gonorrhoeae* testing at HUSLAB, Helsinki, Finland in 2008 (I). The 414 samples were *C. trachomatis* NAAT positive with the Aptima Combo 2 Assay (Hologic). The 55 samples were *C. trachomatis* NAAT positive with the Cobas TaqMan CT Test v2.0 (Roche Diagnostics) able to detect the nvCT, and 26 samples were *C. trachomatis* NAAT negative with the Cobas TaqMan CT Test (Roche Diagnostics) unable to detect the nvCT.

For the detection of the *C. trachomatis* LGV types, we included 140 *C. trachomatis* NAAT positive extragenital samples sent for *C. trachomatis* and *N. gonorrhoeae* testing at HUSLAB, Helsinki, Finland with the Aptima Combo 2 Assay (Hologic) in 2009–2011 (II). The material consisted of 84 rectal (70 from men and 14 from women) and 56 pharyngeal swabs (27 from men and 29 from women) collected with the Aptima Unisex Specimen Collection Kit for Endocervical and Male Urethral Swab Specimens (Hologic).

The study for the transcriptional expression of *C. trachomatis* included 127 cervical swab samples obtained from patients who attended the outpatient STI clinic of Helsinki University Hospital, Helsinki, Finland in 2009–2011 because of symptoms, for follow-up, or because of notification by an infected partner (III). The samples collected with the Aptima Unisex Specimen Collection Kit for Endocervical and Male Urethral Swab Specimens (Hologic) were tested for *C. trachomatis* and *N. gonorrhoeae* with the Aptima Combo 2 Assay (Hologic) at HUSLAB, Helsinki, Finland. The cervical samples collected in Universal Transport Medium (UTM, Copan, Brescia, Italy) were used for the culture of

C. trachomatis. Of the 127 samples, 50 were *C. trachomatis* NAAT positive and 77 were *C. trachomatis* NAAT negative. The *in vivo C. trachomatis* gene expression analysis was performed with the 44 cervical swabs available. The *in vitro* transcriptional expression of *C. trachomatis* was analysed in five low-passage-number (passage ≤5) clinical isolates representing the most common genotypes E and F (E127, E129, E142, F175 and F213) in urogenital samples.

The prevalence of HSV, HHV-6, HPV, *M. genitalium*, and mutations leading to macrolide resistance in *M. genitalium* was investigated in 314 urogenital samples from 157 *C. trachomatis* NAAT positive women, and their age-, location- and time-matched 157 *C. trachomatis* NAAT negative controls participating in an HPV vaccination trial and simultaneously in a *C. trachomatis* screening trial in 2010–2017 (IV). The self-collected vaginal swab samples rinsed in FVU collected with the Abbott multi-Collect Specimen Collection Kit were analysed for *C. trachomatis* and *N. gonorrhoeae* with the Abbott RealTime CT/NG assay (Abbott Molecular) at Fimlab Laboratories, Tampere, Finland. All women with a *C. trachomatis* NAAT positive sample were invited to give a TOC sample one month later. The urogenital samples had previously been analysed for HPV DNA at the Department of Clinical Microbiology, Skåne University Hospital, Malmö, Sweden (Söderlund-Strand et al. 2009; Söderlund-Strand & Dillner 2013).

The collection of the clinical samples was approved by the Independent Institutional Review Board of the Hospital District of Helsinki and Uusimaa (18.2.2009 HUSLAB §19/2009, 15.6.2009 HUSLAB §44/2009, 16.2.2012 HUSLAB §18/2012, 10.1.2013 HUSLAB §2/2013, 11.4.2014 HUSLAB §13/2014, 17.3.2015 HUSLAB §7/2015) (I, II & III), the Ethics Committee of the Department of Medicine, Hospital District of Helsinki and Uusimaa (5.6.2009 §241/2009, 19.11.2012 §295/2012, 18.3.2015 §56/2015) (I, II & III), and the Ethical Review Board of the North Ostrobothnia Hospital District (19.11.2012; §295/2012) (IV). A written informed consent was obtained from the patients (III & IV). A summary of all the clinical samples and the analyses performed in studies I–IV is presented in Table 4.

Table 4. The clinical samples and the analyses performed in studies I–IV.

Study	Samples (n)	Sample type	Collection year	Ctr NAAT result	Analysis method
I	160	FVU, cervical/ vaginal and conjunctival swabs	2008	160 positives	Ctr *ompA* PCR
	495	FVU, cervical/ vaginal swab	2008	469 positives 26 negatives	Swedish nvCT PCR, plasmid sequencing
II	140	rectal and pharyngeal swabs	2009–2011	140 positives	Ctr *pmpH* PCR, Ctr *ompA* PCR and *ompA* sequencing
III	127	cervical swab	2009–2011	50 positives 77 negatives	Ctr culture in McCoy, Ctr infection in HeLa229, Ctr *cpaf*, *tarp*, *tox* and *ompA* RT-PCRs
IV	314	urogenital	2010–2017	157 positives 157 negatives	*M. genitalium*, *M. genitalium* macrolide resistance, HSV, HHV-6 and Ctr *ompA* PCRs

Ctr, *Chlamydia trachomatis*; NAAT, nucleic acid amplification test; FVU, first-void urine; *ompA*, major outer membrane protein gene; PCR, polymerase chain reactions; nvCT, new variant of *C. trachomatis*; *pmpH*, polymorphic membrane protein H gene; *cpaf*, chlamydial protease-like activity factor; *tarp*, translocated actin-recruiting phosphoprotein gene; *tox*, chlamydial cytotoxin gene; RT-PCR, reverse transcriptase-PCR; HSV, herpes simplex virus; HHV-6, human herpesvirus 6

4.2 CELL LINES (I, II, III, IV)

The McCoy cells (from Pekka Saikku, University of Oulu, Finland) were maintained in BKH-21 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 20 μg/ml gentamicin in a humidified incubator with 5% CO_2 at 35 °C (I, II, III & IV).

The HeLa229 cells (CCL-2.1) from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in α-MEM (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine (GlutaMax, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 20 μg/ml gentamicin and 0.5% glucose in a humidified incubator with 5% CO_2 at 35 °C (III).

4.3 *C. TRACHOMATIS* INFECTION OF THE MCCOY AND HELA229 CELLS (I, II, III, IV)

The McCoy cells in 24-well plates were infected and centrifuged at 3000 x g at 30 °C for one hour (I, II, III, IV). BHK-21 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 20 μg/ml gentamicin, 50 U/ml nystatin, 100 μg/ml vancomycin, and 0.5 μg/ml cycloheximide was added, and the plates were incubated in 5% CO_2 at 35 °C for 48 hours. After incubation, the cells were collected in sucrose-phosphate-glutamate, pH 7.2, and slowly frozen to -70 °C.

The HeLa229 cells in 24-well plates were infected at a multiplicity of infection of 1.0 (III). The plates were centrifuged at 3000 x g at 30 °C for one hour and incubated in 5% CO_2 at 35 °C for one hour. α-MEM (Sigma-Aldrich) supplemented with 10% fetal calf serum, 2 mM glutamine (GlutaMax, Gibco, Thermo Fisher Scientific), 20 μg/ml gentamicin, 0.5% glucose, 50 U/ml nystatin and 0.5 μg/ml cycloheximide was added, and the plates were incubated in 5% CO_2 at 35 °C for 2, 6, 12, 24 and 48 hours. After incubation, the cells were collected in ice-cold phosphate-buffered-saline.

4.4 IMMUNOFLUORESCENCE MICROSCOPY (I, III)

Direct immunofluorescence staining was performed on *C. trachomatis* infected cell cultures with a Pathfinder *Chlamydia* Culture Confirmation System (Bio-Rad, Hercules, CA, USA).

4.5 *C. TRACHOMATIS* ISOLATES AND REFERENCE STRAINS (I, II, III, IV)

Study III included 40 low-passage-number (passage ≤5) *C. trachomatis* clinical isolates propagated in McCoy cells. In addition, *C. trachomatis* reference strains types A–K and L2 (ATCC numbers A:VR-571B, B:VR-573, C:VR-572, D:VR-885, E:VR-348B, F:VR-346, G:VR-878, H:VR-879, I:VR-880, J:VR-886, K:VR-887, and L2:VR-903) propagated in McCoy cells were used (I, II, III & IV).

4.6 AMPLIFICATION AND QUANTIFICATION CONTROLS FOR REAL-TIME PCR (I, II, III, IV)

The sequence flanking the deletion site in the plasmid of the Swedish nvCT was cloned into a pCR-Blunt II-TOPO vector with the Invitrogen Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific) to serve as an amplification control in the Swedish nvCT PCR (I). The sample containing the Swedish nvCT was a gift from Björn Herrmann, University of Uppsala, Sweden. To quantify the *C. trachomatis* genome equivalents (GEs) during HeLa229 cell infection, a pIDTSMART-AMP plasmid containing the *C. trachomatis* type E *ompA* PCR target sequence was purchased from Integrated DNA Technologies (Coralville, IA, USA) (III). As an amplification control for HSV, HHV-6 and *M. genitalium* PCR, the PCR target sequences in a pIDTSMART-AMP plasmid were purchased from Integrated DNA Technologies (IV). DNA extracted from *C. trachomatis* reference strain types A–K and L2 (ATCC) propagated in McCoy cells was used as an amplification control in the PCR analyses (I, II, III, IV).

4.7 NUCLEIC ACID EXTRACTION AND CDNA SYNTHESIS (I, II, III, IV)

DNA was extracted from a sample volume of 400 µl of urogenital sample with a MagNA Pure Compact instrument (Roche Molecular Systems, Pleasanton, CA, USA) using a MagNA Pure Compact Nucleic Acid Isolation Kit I and eluted in 50 µl (I for *ompA* PCR, II). DNA was extracted from a sample volume of 200 µl of urogenital sample with a MagNA Pure LC instrument (Roche Molecular Systems) using a MagNA Pure LC DNA Isolation Kit I and eluted in 100 µl (I for nvCT PCR). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) (I). DNA was extracted from 100 or 200 µl of *C. trachomatis* infected McCoy cells with a DNeasy blood and tissue kit (Qiagen) and eluted in 200 µl (III). DNA was extracted from 250 µl of *C. trachomatis* infected HeLa229 cells with a PureLink Genomic DNA Kit (Thermo Fisher Scientific) and eluted in 50 µl (III). DNA was extracted from a sample volume of 500 µl of urogenital sample with a MagNA Pure LC 2.0 System (Roche Molecular Systems) using a MagNA Pure LC DNA Isolation Kit-Large Volume and eluted in 100 µl (IV).

RNA was extracted from 250 µl of *C. trachomatis* infected HeLa229 cells stored in RNAlater Solution (Thermo Fisher Scientific) with an RNAqueous-4PCR Kit (Thermo Fisher Scientific) and treated with deoxyribonuclease using a TURBO-DNA-free Kit (Thermo Fisher Scientific) (III). RNA was extracted from the cervical samples with an RNAqueous-4PCR Kit (Thermo Fisher Scientific) and eluted in 50 µl (III).

DNA and RNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA was stored in -20 °C or -70 °C, and RNA was stored in -70 °C until analysis (I, II, III).

Complementary DNA (cDNA) was synthesised using the RNA extracted from infected HeLa229 cells and cervical samples with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) (III).

4.8 DETECTION OF NUCLEIC ACIDS (I, II, III, IV)

For the *C. trachomatis ompA* genotyping, two primer sets and eleven genotype-specific probes for types D–K and L1–L3 were used with a method described previously (Jalal et al. 2007) (I). For detecting the Swedish nvCT, primers and a probe flanking the deletion sequence in the cryptic plasmid of *C. trachomatis* were used employing a method developed earlier (Catsburg et al. 2007) (I). Both PCRs contained 250 nM primers and 100 nM probes.

A previously described method was used to detect the *C. trachomatis* LGV types (Chen et al. 2008) based on a 36 base pair deletion in the sequence of the *pmpH* gene, which is present only in LGV strains (Stothard et al. 2003) (II). The method included primers and two probes for detecting both LGV (L1–L3) and non-LGV (D–K) types. The PCRs contained 600 nM primers and 100 nM probes.

The primers and probes for the detection of the *C. trachomatis cpaf*, *tarp* and *tox* genes were designed with the Primer Express software v3.0 (Applied Biosystems, Thermo Fisher Scientific), based on the *C. trachomatis* complete genome sequences in the NCBI Reference Sequence Database (Table 5) (III). The primers and probes were tested for specificity with the NCBI's basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/) and with *C. trachomatis* reference strains. The PCR for *cpaf* contained 600 nM primers and 200 nM probes, for *tarp* the PCR contained 300 nM primers and 200 nM probes, and for *tox* the PCR contained 600 nM primers and 100 nM probes. The *ompA* PCR was performed with a method designed earlier and contained 250 nM primers and 100 nM probes (Jalal et al. 2006). Each time after infection the raw *ompA*, *cpaf*, *tarp* and *tox* PCR amplification data were normalised against the number of *C. trachomatis* GEs in each sample, determined with the *ompA* PCR (Borges et al. 2010).

Table 5. Sequences of the *C. trachomatis cpaf*, *tarp* and *tox* primers and probes (III).

Ctr target gene	Amplicon length (bp)		Primer/probe sequence (5´–3´)
cpaf	86	forward	TAGGATGGGATCTTGTTCAAAGCT
		reverse	CTGCTGGCAAAAACTTGTTGAT
		probe	6-FAM-CTGCACAGCAGAAGCTTCGTACACAAGAA-BHQ-1
tarp	108	forward	CCTCTTCTGGAGATGATTCAGGAA
		reverse	TACGCACGGCAGAAAGGATA
		probe	6-FAM-CCTCTGTCGGAAATGACGGACCTGCT-BHQ-1
tox	106	forward	GATTCTTTAATTTCTGCTTGCTGAAA
		reverse	TGTTCGATCTCCTCAGTAGGAAGTTT
		probe	6-FAM-CTCGGCAATATCAATGACGAAACGCGT-BHQ-1

Ctr, *Chlamydia trachomatis*; bp, base pairs; *cpaf*, chlamydial protease-like activity factor; FAM, fluorescein amidite; BHQ, black hole quencher; *tarp*, translocated actin-recruiting phosphoprotein gene; *tox*, chlamydial cytotoxin gene

The PCRs for the detection of HSV (HSV-1 and HSV-2 DNA polymerase gene) (Pillet et al. 2015), HHV-6 (HHV-6A and HHV-6B U67 gene) (Karlsson et al. 2012), *M. genitalium* (adhesin gene) (Jensen et al. 2004), *M. genitalium* macrolide resistance (mutations in the 23S rRNA gene) (Nummi et al. 2015) and human beta-globin gene (Nummi et al. 2015) were developed previously (IV). The PCR for HSV contained 400 nM primers and 160 nM probes, for HHV-6 the PCR contained 100 nM forward primers, 600 nM reverse primers and 200 nM probes, for *M. genitalium* the PCR contained 100 nM primers and 100 nM probes, for *M. genitalium* macrolide resistance the PCR contained 50 nM forward primers, 400 nM reverse primers and 150 nM probes, and for beta-globin the PCR contained 200 nM primers and 100 nM probes.

CRB were detected with Pan-*Chlamydiales* PCR (Lienard et al. 2011) with modifications (Hokynar et al. 2016). Pan-*Chlamydiales* PCR amplifies an approximately 200 bp fragment of the 16S rRNA gene and detects a wide range of different members of the *Chlamydiales* order. The PCR contained 100 nM primers and 100 nM probes.

The primers and probes were purchased from Applied Biosystems (Thermo Fisher Scientific), Integrated DNA Technologies, Metabion International AG, Oligomer and TAG Copenhagen A/S. The PCRs were performed in a 25 µl volume containing 12.5 µl Maxima Probe qPCR Master Mix (Thermo Fisher Scientific), Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific)

or TaqMan Universal Master Mix (Applied Biosystems, Thermo Fisher Scientific). Real-time PCR analyses were performed with an ABI 7500 instrument and Sequence Detection Software v1.3.1 (Applied Biosystems, Thermo Fisher Scientific) or Rotor-Gene 6000 (Qiagen). The thermal cycling conditions were two minutes at 50 °C, ten minutes at 95 °C, 40 or 45 cycles of 15 seconds at 95 °C, and one minute at 60 °C.

4.9 DNA SEQUENCING (I, II)

The samples containing the Swedish nvCT and the *C. trachomatis* L2 type were sequenced at the Biomedicum Sequencing Unit, Faculty of Medicine, University of Helsinki, Finland. For the Swedish nvCT, NCBI's Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design sequencing primers for the region flanking the deletion site of the variant plasmid based on the sequence of the plasmid (GenBank no. EF121575) (I). The part of the *C. trachomatis ompA* gene including the nucleotide mismatches between the different L2 types (L2, L2a and L2b) (Spaargaren, Fennema, et al. 2005) of the L2 positive samples was sequenced with primers P1 and P2 (Jalal et al. 2007) (II). Sequencing of the PCR products was performed in both directions. The sequences were analysed with Sequence Scanner Software v1.0 (Applied Biosystems, Thermo Fisher Scientific) and compared to the sequence database with NCBI's Nucleotide BLAST.

4.10 STATISTICAL ANALYSIS (IV)

The *p*-values were calculated with the Fisher's exact test using the IBM SPSS Statistics v24 (IBM, Armonk, NY, USA). A *p*-value less than 0.05 was considered statistically significant. The 95% confidence intervals were also calculated (Lane 2019).

5. RESULTS

5.1 *C. TRACHOMATIS OMPA* GENOTYPE DISTRIBUTION IN THE UROGENITAL SAMPLES (I, III, IV)

Of the 160 *C. trachomatis* NAAT positive urogenital samples collected and analysed in 2008, 144 (90%) could be genotyped with the *ompA* PCR using genotype-specific probes for types D–K (I) (Table 7). The genotype distribution was: E 40% (n=57), F 28% (n=41), G 13% (n=19), D 8% (n=11), K 5% (n=7), H 3% (n=5), J/Ja 2% (n=3), I/Ia 0% (n=0) and D-F mixed infection 1% (n=1). The 16 samples that remained negative for genotypes D–K were analysed for genotypes L1–L3. We did not detect any L types among these urogenital samples. The remaining 16 (10%) samples most probably did not include enough DNA to be genotyped.

We were able to culture *C. trachomatis* from 40 (80%) cervical samples from 50 *C. trachomatis* NAAT positive patients collected in 2009–2011 (III). The *C. trachomatis ompA* genotype distribution of the isolates was E 40% (n=16), F 20% (N=8), G 13% (n=5), D 10% (n=4), K 10% (n=4), B 5% (n=2) and H 2.5% (n=1). Our genotyping results have been confirmed with WGS and the data have been submitted to the ENA (Hadfield et al. 2017).

Of the *C. trachomatis* NAAT positive urogenital samples collected in 2010–2017 from 166 infection episodes of 157 women, 146 (88%) samples could be genotyped and 20 (12%) samples were non-typeable (IV). The genotype distribution was E 47% (n=69), F 27% (n=39), G 9% (n=13), K 8% (n=11), D 5% (n=8), H 3% (n=4) and J 1% (n=2). The genotype distributions from studies I, III and IV are presented in Table 9 in Discussion.

5.2 PREVALENCE OF THE *C. TRACHOMATIS* LGV TYPES AND THE *OMPA* GENOTYPE DISTRIBUTION IN THE EXTRAGENITAL SAMPLES (II)

Of the 140 *C. trachomatis* NAAT positive rectal and pharyngeal samples collected in 2009–2011, 114 (81%) samples (85% of the rectal and 77% of the pharyngeal swabs) could be genotyped as LGV (L1–L3) and non-LGV (D–K) types with the *pmpH* PCR (Table 6). The remaining 26 (19%) samples were non-typeable using this method, most probably due to the low quantity of chlamydial DNA in the samples. Non-LGV types were detected in 104 (91%) samples, LGV types were detected in nine (8%) samples and one sample (1%) contained both non-LGV and LGV types.

LGV types were detected in nine rectal swabs and one pharyngeal swab. One patient had LGV types both at the rectal and the pharyngeal sites, so altogether nine LGV DNA positive patients were identified.

Table 6. The non-LGV and LGV types detected in rectal and pharyngeal samples with the *pmpH* PCR

Genotype	Rectal samples n (%)	Pharyngeal samples n (%)	All samples n (%)
Non-LGV	62 (87)	42 (98)	104 (91)
LGV	8 (11)	1 (2)	9 (8)
Non-LGV & LGV	1 (1)	0 (0)	1 (1)
Total	**71 (100)**	**43 (100)**	**114 (100)**

LGV, lymphogranuloma venereum; *pmpH*, polymorphic membrane protein H gene; PCR, polymerase chain reaction

The 140 *C. trachomatis* NAAT positive extragenital samples were genotyped with the *ompA* PCR. Of the 140 samples, 104 (74%) (71% of the rectal and 79% of the pharyngeal swabs) could be genotyped and 36 (26%) remained without a genotype (Table 7). All the samples positive for LGV with the *pmpH* PCR were of genotype L2 with the *ompA* PCR. In the rectal swabs of MSM, genotypes G, D, E and L2 were most frequently detected, while in heterosexual women E and D were the most frequent genotypes. In the pharyngeal samples, genotypes E and D were the most detected in men, and genotypes E and F in women. In addition, the *ompA* gene of the samples positive for genotype L2 was sequenced. We identified six samples with genotype L2b and two samples with genotype L2. Because of an L2-J mixed infection, one sample could not be sequenced.

Table 7. The *C. trachomatis ompA* genotype distribution of rectal and pharyngeal samples in men and women (II), and the genotype distribution of urogenital samples (I).

	Study II (2009–2011)				Study I (2008)
	Men		Women		Men and women
Genotype	Rectum n (%)	Pharynx n (%)	Rectum n (%)	Pharynx n (%)	Urogenital n (%)
E	8 (16)	10 (53)	5 (45)	10 (40)	57 (40)
F	0 (0)	1 (5)	1 (9)	5 (20)	41 (28)
G	15 (31)	1 (5)	0 (0)	3 (12)	19 (13)
D	13 (27)	6 (32)	2 (18)	1 (4)	11 (8)
K	0 (0)	0 (0)	0 (0)	1 (4)	7 (5)
H	0 (0)	0 (0)	1 (9)	2 (8)	5 (3)
J	3 (6)	0 (0)	0 (0)	1 (4)	3 (2)
I	1 (2)	0 (0)	1 (9)	0 (0)	0 (0)
L2	8 (16)	1 (5)	0 (0)	0 (0)	0 (0)
Mixed	1 (2)	0 (0)	1 (9)	2 (8)	1 (1)
Total	**49**	**19**	**11**	**25**	**144**

Nine LGV positive patients were identified. The mean age of the patients was 37 (range 22–52), all patients were MSM, and all except one were HIV positive. Other STIs, including syphilis (4/9), gonorrhoea (3/9) and HCV (1/9) were detected among these patients. Five of the nine patients reported sexual contacts outside Finland in other European countries, and several of the patients had had more than ten sexual partners during the last 12 months. *C. trachomatis* was not detected in the FVUs or urethral samples of these patients taken at the same visit as the rectal swab. Two of the patients also had a *C. trachomatis* positive pharyngeal swab. One of the pharyngeal swabs contained the same genotype L2, the other could not be genotyped.

5.3 PREVALENCE OF THE SWEDISH NEW VARIANT OF *C. TRACHOMATIS* IN THE UROGENITAL SAMPLES (I)

Of the 469 urogenital samples screened for the Swedish nvCT, two samples (0.4%) were identified with the variant plasmid. The samples were from two female patients, one was FVU and the other was a vaginal swab sample. Sequencing confirmed that the *C. trachomatis* in the samples harboured the variant plasmid, as the sequence flanking the deletion region in the ORF1 was identical to that of the Swedish nvCT (Ripa & Nilsson 2006). Both nvCTs were of genotype E, as has been reported in

other countries (Dahlberg et al. 2018). The samples were originally tested positive with the Aptima Combo 2 Assay (Hologic) that can detect the Swedish nvCT as the test targets 23S rRNA of *C. trachomatis*.

5.4 PREVALENCE OF HSV, HHV-6, HPV AND *MYCOPLASMA GENITALIUM* IN THE UROGENITAL SAMPLES (IV)

The prevalence of HSV, HHV-6, HPV and *M. genitalium* was analysed in the urogenital samples of 314 young women (157 *C. trachomatis* NAAT positive women and their 157 *C. trachomatis* NAAT negative controls). HPV DNA was frequently (46%) detected in the urogenital samples, and more frequently detected among the *C. trachomatis* positives than among the negatives (66% vs. 25%, p<0.001) (Table 8). HSV DNA was present in 1% of the samples, HHV-6 DNA in 12% of the samples, and *M. genitalium* DNA in 3.2% of the samples. Of the ten samples positive for *M. genitalium* DNA, two samples (20%) contained a mutation associated with macrolide resistance. A slightly higher prevalence of *M. genitalium* (4.5% vs. 1.9%) and HSV DNA (1.9% vs. 0%) in the *C. trachomatis* positive samples was observed, but these differences were not statistically significant. Among the 314 specimens, 22 (21 among *C. trachomatis* NAAT positive and one among *C. trachomatis* NAAT negative) were positive for three of the microbes analysed. Only one sample was positive for *N. gonorrhoeae*.

Among the 157 *C. trachomatis* NAAT positive women, 166 infection episodes were evaluated, as nine women had two *C. trachomatis* infection episodes during the study period. A TOC sample was taken approximately one month after the treatment with a single 1 g dose of azithromycin, and it was available from 121 (73%) women. The infection episodes were divided into infection not cleared (*C. trachomatis* TOC positive, n=16) and infection cleared (*C. trachomatis* TOC negative, n=105) based on the *C. trachomatis* NAAT result of the TOC sample. Of the 121 TOC samples, sixteen (13%) were positive for *C. trachomatis*. A TOC sample was not available from 45 (27%) women. The prevalence of HSV, HPV and *M. genitalium* was similar between those who cleared the *C. trachomatis* infection and those who did not (Table 8). HHV-6 DNA was slightly more frequently detected among those who gave a *C. trachomatis* positive TOC sample (19% vs. 10%), but this difference did not reach statistical significance. No differences were observed between the *C. trachomatis ompA* genotype distributions of those who cleared the infection and those who did not.

Table 8. The prevalence (% and 95% confidence interval) of HSV, HHV-6, HPV and *M. genitalium* DNA in the urogenital samples of 157 *C. trachomatis* NAAT positive and 157 NAAT negative women, and in the samples of women who gave a *C. trachomatis* test-of-cure (TOC) positive (n=16) and a TOC negative (n=105) negative sample.

	HSV	HHV-6	HPV	*M. genitalium*
Ctr positive (n=157)	3 (1.9%; 0–4.3%)	17 (11%; 5.6–16.0%)	104 (66%; 58.5–73.9%)	7 (4.5%; 1.0–8.0%)
Ctr negative (n=157)	0 (0%)	22 (14%; 8.3–19.7%)	39 (25%; 17.7–31.9%)	3 (1.9%; 0–4.3%)
p-value[1]	0.248	0.494	<0.001	0.336
Total (n=314)	3 (1.0%)	39 (12%)	143 (46%)	10 (3.2%)
TOC positive (n=16)	0 (0%)	3 (19%; 0–41.1%)	9 (56%; 28.9–83.7%)	0 (0%)
TOC negative (n=105)	1 (1.0%; 0–3.4%)	10 (10%; 3.4–15.6%)	75 (71%; 62.4–80.5%)	5 (4.8%)
p-value[1]	1.000	0.377	0.250	1.000

HSV, herpes simplex virus; HHV-6, human herpesvirus 6; HPV, human papillomavirus; Ctr, *Chlamydia trachomatis*; TOC, test-of-cure; [1] Fisher's exact test was used to calculate the statistical significance.

5.5 PREVALENCE OF *CHLAMYDIA*-RELATED BACTERIA IN THE UROGENITAL SAMPLES

The *C. trachomatis* NAAT negative urogenital samples from study IV (n=157) and 82 additional *C. trachomatis* NAAT negative urogenital samples from the *C. trachomatis* screening trial were analysed for CRB with the Pan-*Chlamydiales* PCR. This PCR detects all members of the *Chlamydiales* order, so we could clearly not analyse the samples positive for *C. trachomatis*. The amplicons from PCR positive reactions were sequenced and analysed with BLAST to verify the PCR findings and to classify the detected CRB. Of the 239 samples analysed, only two (0.8%) contained CRB DNA (Hokynar et al. unpublished results). The first sample contained a member of *Parachlamydiaceae* which was highly similar (97%) to the CRB sequences detected in *Ixodes ricinus* ticks (Pilloux et al. 2015). The second sample contained a CRB resembling those detected in nasopharyngeal samples of hospitalised children in Switzerland (Lienard et al. 2011). *W. chondrophila* has a sero-epidemiological association with miscarriage (Baud et al. 2014), but this bacterium was not detected among the samples from young women.

5.6 EXPRESSION OF THE *C. TRACHOMATIS OMPA, CPAF, TARP* AND *TOX* GENES (III)

5.6.1 GENE EXPRESSION OF *C. TRACHOMATIS* IN THE CERVICAL SWABS

The *in vivo* expression of the *C. trachomatis ompA*, *cpaf*, *tarp* and *tox* genes was first analysed in the cervical swabs. Among the 44 *C. trachomatis* NAAT positive samples available for analysis, small amounts of messenger ribonucleic acid (mRNA) of one or several of the investigated genes were observed in nine samples: *ompA* mRNA in five, *cpaf* mRNA in five, *tarp* mRNA in three, and *tox* mRNA in three samples. Among the eight *C. trachomatis* NAAT negative samples, no such mRNA was detected.

5.6.2 GROWTH OF *C. TRACHOMATIS* CLINICAL ISOLATES AND REFERENCE STRAINS

The HeLa229 cell cultures were infected with the five low-passage-number *C. trachomatis* clinical isolates (E127, E129, E142, F175 and F213) representing the most common genotypes E and F and the *C. trachomatis* reference strains type E and F. The number of *C. trachomatis* GEs was determined with the *ompA* PCR at 2, 6, 12, 24 and 48 hpi. The results are based on two biological replicates, and the experiment was performed twice with similar results. The exponential period of growth in the reference strains E and F was observed at 12–36 hpi (Figure 4). In the clinical isolates, the exponential period of growth took place at 12–24 hpi in E127, at 12–36 hpi in E142, F213 and F175, and at 24–36 hpi in E129.

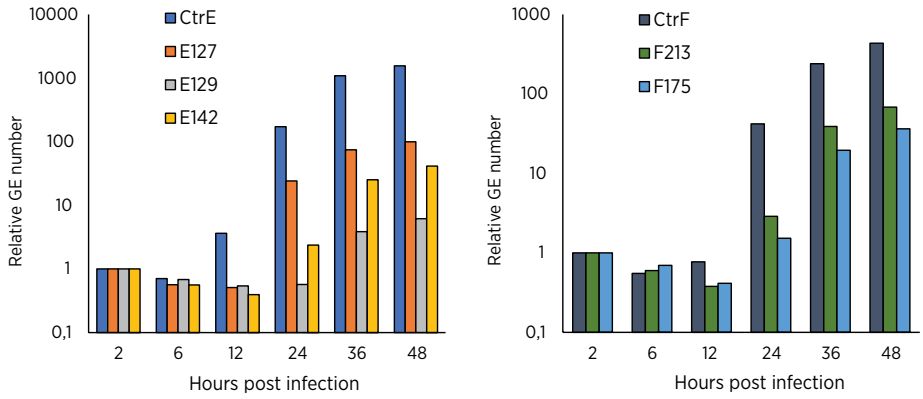

Figure 4. Growth kinetics of *C. trachomatis* reference strain E (CtrE) and clinical isolates E127, E129 and E142 (left panel), and *C. trachomatis* reference strain F and clinical isolates F213 and F175 (right panel) in HeLa229 cells at 2, 6, 12, 24 and 48 hours post infection determined with the major outer membrane protein gene (*ompA*) PCR. The results are shown as relative genome equivalents (GEs), the number of GEs at 2 hpi was set as one. The results are based on two biological replicates, and the experiment was performed twice with similar results.

The generation times were calculated by comparing the number of GEs at the beginning and the end of the exponential growth phase of *C. trachomatis*. We observed a doubling time of 2.8 h for *C. trachomatis* reference strain type E, a doubling time of 2.9 h for reference strain type F, and a doubling time of 2.2 h for the clinical isolate E127. The doubling times were longer (E129 4.3 h, E142 4.0 h, F213 3.6 h and F175 4.3 h) in the other clinical isolates. All clinical isolates, except one (E127), exhibited a slower growth rate than the reference strains.

After the lag phase (6–12 hpi) of the chlamydial developmental cycle, we observed a 2200-fold increase in the number of *C. trachomatis* GEs in the *C. trachomatis* reference strain type E and a 790-fold increase in GEs in the reference strain type F. For the clinical isolates, the increase was more modest, with an 11–190-fold increase in types E, and a 90–180-fold increase in types F. The reference strain types E and F were more efficient in producing progeny than the clinical isolates.

5.6.3 GENE EXPRESSION OF *C. TRACHOMATIS* IN THE HELA229 CELLS

The expression of the *C. trachomatis ompA*, *cpaf*, *tarp* and *tox* genes was studied in HeLa229 cells infected with the five low-passage-number clinical isolates (E127, E129, E142, F175 and F213) and the reference strains type E and F at 2, 6, 12, 24, 36

and 48 hpi. The results are based on two biological replicates, and the experiment was performed twice with similar results.

The *C. trachomatis ompA* was expressed between 12 and 48 hpi, and the peak of the expression was observed at 24 hpi in the reference strains and the clinical isolates (Figure 5). Among the reference strains and the clinical isolate E127, the *ompA* expression was high already at 12 hpi compared with the other clinical isolates. The expression of *cpaf* peaked at 12 hpi in the clinical isolate E127, and at 24 hpi in the other clinical isolates and the reference strains. In addition, the *cpaf* expression in the reference strain type E was high at 6, 12 and 24 hpi and in the reference strain type F at 24 hpi, compared to the clinical isolates. The chlamydial *tarp* was expressed between 24 and 48 hpi and the peak of expression was between 36 and 48 hpi among all clinical isolates and the reference strains. The expression of *tox* was observed between 12 and 48 hpi, and the peak of expression was at 12 hpi for the reference strains and the clinical isolate E127, and at 24 hpi for the other clinical isolates.

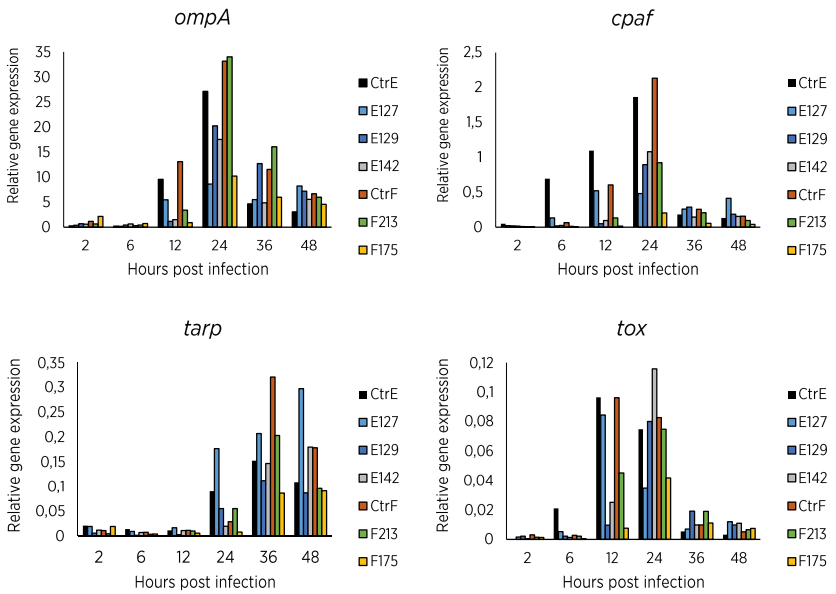

Figure 5. The expression of the *C. trachomatis* major outer membrane protein (*ompA*), the chlamydial protease-like activity factor (*cpaf*), the translocated actin-recruiting phosphoprotein (*tarp*) and the chlamydial cytotoxin (*tox*) genes of the reference strains E (CtrE) and F (CtrF), and the clinical isolates E127, E129, E142, F213 and F175 in the HeLa229 cells at 2, 6, 12, 24 and 48 hours post infection. At each timepoint, raw *ompA*, *cpaf*, *tarp* and *tox* PCR amplification data were normalised against the number of *C. trachomatis* genome equivalents (GEs) in each sample (Borges et al. 2010). The results are based on two biological replicates, and the experiment was performed twice with similar results.

6. DISCUSSION

6.1. *C. TRACHOMATIS OMPA* GENOTYPE DISTRIBUTION (I, II, III, IV)

Before study I, the Finnish *C. trachomatis ompA* genotype distribution among urogenital samples was analysed in 1987 (Saikku & Wang 1987) and 1996 (Penttilä et al. 1996). When the distribution of the different genotypes from study I is compared to those from 1987 and 1996, the proportion of genotypes F and G seems to have increased, whereas the proportion of genotypes D and E seems to have remained fairly stable (Table 9). However, diverse typing methods were used in these studies: monoclonal antibodies in 1987, *ompA* PCR and RFLP in 1996, and *ompA* PCR and genotype-specific probes in study I. It is of interest that a similar change was observed with a method based on monoclonal antibodies in Seattle, USA: the proportion of infections due to serovars F and G increased in 1988–1996 (Suchland et al. 2003). When the genotype distribution from studies I, III and IV was compared, the proportion of genotypes has remained quite stable in Finland during the last ten years. This observation can be considered reliable, as the same method was used for genotyping, although the specimen collections differed in these studies. Studies I and III included samples collected at the Helsinki and Uusimaa region, while in study IV the samples were collected in several communities across Finland. The *ompA* genotype distribution seems to be similar throughout Finland.

When the Finnish *ompA* genotype distribution in study I is compared to those reported in other countries, the proportion of genotypes is fairly similar in Finland, Sweden (Lysén et al. 2004), the Netherlands (Spaargaren et al. 2004) and Portugal (Nunes et al. 2009). In contrast, the proportion of genotypes E and G seems slightly higher in Europe than in the USA, while the proportions of genotype J/Ja and I/Ia tend to be somewhat higher in the USA than in Europe (Millman et al. 2004). The typing methods used in these studies varied which might explain the observed differences. However, when studies using data produced with *ompA* PCR and sequencing are compared (Lysén et al. 2004; Millman et al. 2004; Nunes et al. 2009), the observations remain.

There is conflicting evidence on the connection of the different *ompA* genotypes to the clinical manifestations of *C. trachomatis* infections. Some studies found no association between genotype and disease phenotype (Lysén et al. 2004; Persson & Osser 1993). However, other studies linked genotypes F and/or G to abdominal pain, symptomatic severe endometrial disease or PID (Dean et al. 1995; Gao et al. 2007; Geisler et al. 2003; Millman et al. 2006; van Duynhoven et al. 1998). In

Table 9. The *C. trachomatis ompA* genotype distribution (%) in Finland 1987–2017.

Sero-/genotype	**1987**[a] n=51	**1996**[b] n=122	**2008** (I)[c] n=144	**2009–2011** (III)[c] n=40	**2010–2017** (IV)[c] n=144
B	2	2	ND	5	ND
D	6	9	8	10	6
D/B	ND	2	ND	ND	ND
E	25	33	40	40	47
ED	25	ND	ND	ND	ND
Total (B-complex)	58	46	48	55	53
F	10	22	28	20	27
G	4	13	13	13	9
K	8	2	5	10	8
Total (Intermediate complex)	22	37	46	43	44
C	0	ND	ND	0	ND
C/J	ND	6	ND	0	ND
C-group	ND	8	ND	0	ND
H	6	3	3	3	3
I	6	ND	0	0	1
J/Ja	8	ND	2	0	0
Total (C-complex)	20	17	5	3	4

[a] Micro-immunofluorescence (MIF) (Saikku & Wang 1987)
[b] *C. trachomatis ompA* PCR and restriction length polymorphism (RFLP) (Penttilä et al. 1996)
[c] *C. trachomatis ompA* real-time PCR and genotype-specific probes (I, III, IV)
C-group: genotypes A, C, H, I, Ia, J, K and L3; ND, not determined

addition, genotype E was associated with asymptomatic infections (Dean et al. 1995; Gao et al. 2007; Sturm-Ramirez et al. 2000). If infections caused by genotype E show fewer symptoms than infections due to the other genotypes, *C. trachomatis* genotype E might be able to spread without being detected, which could explain the high proportion of infections caused by this genotype. The opposing results in the studies might be due to the small number of cases investigated, confounding factors such as age and race, lack of specific diagnostic criteria and differences in study design. Previously, it was shown that there is extensive recombination in the *ompA* gene which could lead to contradicting observations on the relationship of the *ompA* genotypes to infection outcome (Harris et al. 2012). However, a recent study showed that genotype E, as the most epidemiologically successful genotype, has

emerged worldwide relatively recently and has not been affected by recombination in the *ompA* gene (Hadfield et al. 2017). Other biological characteristics of *C. trachomatis* and host immunity have most probably more impact on the outcome of *C. trachomatis* infections than the *ompA* genotype alone.

In study II, the rectal specimens from both MSM and heterosexual women were included. The genotype distribution in the rectal samples among MSM differed from the proportion of genotypes in the rectal samples among women, and from the genotype distribution in the urogenital samples (study I) (Table 7). The four most common genotypes in rectal samples from MSM were G, D, E and L2. This genotype distribution is similar to that from other studies on MSM (Bax et al. 2011; Geisler et al. 2008; Isaksson et al. 2017). Among the rectal samples in women, genotypes E and D were the most frequently detected. This corresponds well with an earlier study, where women were more often infected with genotype E and less often with genotype G than MSM at rectal sites (Geisler et al. 2008). It seems that the genotype distribution in the rectum samples among women is similar to that in the urogenital samples, but the proportion of genotypes in the rectal specimens among MSM is unique, with genotype G predominating. The rectal specimens from heterosexual women tested more often positive for *C. trachomatis* (13%) than the rectal samples from MSM (7%). This highlights the importance of testing also women in addition to testing MSM at rectal sites. It has been shown that rectal infections among women are more common than the rate of reported anal intercourses (Chan et al. 2016). This could be due to inaccuracies in the self-reported history of sexual practices, but might also be explained by autoinoculation of *C. trachomatis* from the gastrointestinal tract (Rank & Yeruva 2014). It might be of benefit to treat women with doxycycline instead of azithromycin as doxycycline is more effective in clearing rectal *C. trachomatis* infections (Craig et al. 2015). LGV infections have rarely been reported in women (Peuchant et al. 2011; Verweij et al. 2012) and accordingly, only non-LGV genotypes were detected among women in study II. The pharyngeal specimens were from heterosexual men, MSM, and women. The genotype distribution in the pharyngeal samples differed from the proportion of genotypes in the rectal samples among MSM. In the pharyngeal samples, the four most common genotypes were E, D, F and G, in contrast to the most frequently detected genotypes G, D, E and L2 in the rectal samples from MSM. The genotype distribution in the pharyngeal samples seems to be similar to that of the urogenital samples.

The differences in the genotype distributions at the rectal, pharyngeal and urogenital sites can be due to multiple reasons, such as different transmission dynamics, core-group associated factors or tissue tropism. It has been proposed that polymorphisms at certain loci are associated with rectal tropism of *C. trachomatis* serovar G isolates (Jeffrey et al. 2010). It has also been speculated that LGV genotypes

might have a higher affinity to rectal mucosa than to urethral epithelium, but tissue tropism of *C. trachomatis* has not been confirmed (Isaksson et al. 2017; Versteeg et al. 2014). MSM and heterosexuals have their separate sexual networks which most probably lead to different proportions of genotypes at the rectal, pharyngeal and urogenital sites.

6.2 *C. TRACHOMATIS* LGV TYPES (II)

In study II, during the sample collection period between February 2009 and August 2011 at HUSLAB, 8% of the rectal samples tested positive for *C. trachomatis* and 13% of the genotyped samples contained LGV DNA. The *C. trachomatis* positivity in rectal samples was quite similar to the one reported in the UK (Ward et al. 2009). In study II, LGV DNA was rarely (2%) detected in the pharyngeal samples. Of the nine patients with LGV, six were confirmed by *ompA* sequencing to be infected with genotype L2b and two with genotype L2. This corresponded well to the situation in other European countries where genotype L2b has been the causative genotype in the majority of cases, however also L2 genotypes have been identified (Savage et al. 2009). The proportion of infections due to genotypes L2b and L2 seems to be changing, as recently in Sweden LGV caused by L2 was detected more frequently than before, half of the LGV cases being of genotype L2 in 2015 (Isaksson et al. 2017). This information is unfortunately not available from Finland after 2011, as a simplified version of our genotyping method to detect L types was adopted in a clinical laboratory. It does not differentiate the L types, and the LGV positive samples are not routinely sequenced for *ompA*.

Only one LGV positive patient had significant lymphadenopathy, whereas the other patients had a proctitis that was either fairly mild or asymptomatic. This was in contrast to a study performed in the UK, where the majority of rectal LGV infections were symptomatic (Ward et al. 2009). However, in more recent studies performed in the UK and the Netherlands, approximately a quarter of the LGV infections were asymptomatic (de Vrieze et al. 2013; Saxon et al. 2016). In addition, inguinal LGV was rare compared to anal LGV in Amsterdam in 2005–2012 (de Vrieze et al. 2013). As reported before, the LGV positive patients in study II were all MSM, most of them had HIV and other STIs, and the patients were older (over 35 years old) than patients with a urogenital infection (Savage et al. 2009). LGV was not detected in FVU or urethral samples among these LGV positive patients, and only one patient was positive for LGV both at the rectal and pharyngeal sites. Indeed, if MSM are tested at the urogenital sites only, approximately 14–85% of the infections will be missed depending on the study (Chan et al. 2016). The transmission patterns of LGV among MSM are not fully known. As very few cases

of urogenital LGV are reported (de Vrieze et al. 2017), it is proposed that rectum-to-rectum transmission takes place through the use of shared sex toys, group sex, and fisting reported among MSM (de Vries 2019). LGV transmission might also occur through the ano-oral route via oral sex or the use of saliva as a lubricant for anal sex (de Vries 2016). After establishing LGV pharyngitis, *C. trachomatis* might pass through the gastrointestinal tract to the rectum and be transmitted further.

In study II, altogether nine LGV positive patients were identified: three patients in 2009, two patients in 2010 and four patients in 2011. It is not known whether the first LGV case among MSM emerged in Finland in 2009 or earlier. The first two LGV cases in Sweden were reported in 2004 (Berglund et al. 2005), and retrospective analysis carried out in Sweden revealed no additional cases of LGV in 2004–2005 (Klint et al. 2006). We were unfortunately unable to perform a retrospective analysis of rectal samples in Finland. It has been shown that the L2b type causing the LGV outbreak in Europe was already present in San Francisco, USA in the 1980s and was circulating in Amsterdam in 2000 without being detected (Spaargaren, Schachter, et al. 2005).

Since 2011, after introducing an in-house typing test (II) for the *C. trachomatis* NAAT positive specimens into a clinical laboratory, laboratory confirmed LGV infections have been notified by clinicians to the NIDR. By the end of year 2018, 51 LGV cases were reported to the NIDR of which one third was reported in 2018 (NIDR 2019). Of the 51 LGV infections, most were reported among MSM, 20 were acquired in Finland, 18 were acquired in another country in Europe, and for 13 cases the information was not available. The nationality of the LGV patients was Finnish in 39 cases and non-Finnish in 12 cases. Most of the Finnish LGV cases are probably sporadic imports from other European countries. In contrast to the rather low number of notified LGV cases in Finland, almost 2000 cases of LGV were reported in 24 European countries in 2017 (ECDC 2019b). France, the Netherlands, Spain, and the United Kingdom accounted for 86% of all notified LGV cases and in these countries endemic LGV infections occur. As in Finland, almost all cases were reported among MSM and 64% of the patients were HIV positive. The number of reported LGV cases in 2017 decreased by 13 % compared to 2016, which was the first reduction in the number of cases since 2009. This reduction is possibly linked to changes in testing practices rather than the decreased transmission of LGV. Overall, the number of LGV cases reported is likely an underestimation because many countries in Europe do not have specific diagnostic tests or a national surveillance system for LGV.

Urogenital *C. trachomatis* infections are diagnosed with sensitive and specific NAATs, but commercial PCR-based tests validated for the detection of LGV from rectal samples are still unavailable. As LGV infections have previously been rare in the Western countries, they could have been misdiagnosed as, for example,

inflammatory bowel disease (Gallegos et al. 2012) or lymphoma (Crickx et al. 2016). The lack of specific tests combined with unawareness of the disease can lead to delayed diagnosis, inadequate antibiotic treatment and further transmission of the infection. Since 2011, it has been possible to test clinical specimens for LGV at HUSLAB, Helsinki, as a national service, with the in-house PCR method setup in study II upon suspicion of an LGV infection.

6.3 *C. TRACHOMATIS* VARIANTS (I)

Despite our proximity to Sweden, the new variant was rarely detected in Finland, as only two samples (0.4%) contained the nvCT in study I. Unfortunately, both patients were lost from the follow-up, so we were not able to obtain information on the possible travel history or contacts to Sweden. Except for Norway, where the nvCT comprised approximately 3% of the *C. trachomatis* cases detected by a Becton Dickinson test in 2008, the nvCT has seldom been reported or has been reported absent in several countries outside Sweden (Dahlberg et al. 2018). The population affected by the nvCT comprised mainly of young heterosexual individuals with localised sexual networks in Sweden, which may be the reason why the nvCT did not spread significantly outside Sweden.

After the identification of the nvCT and the release of the updated NAATs, the proportion of infections caused by the nvCT decreased significantly in Sweden (Dahlberg et al. 2018). In the counties using the Roche test unable to detect the nvCT, the decrease was from 56% in 2007 to 7% in 2015, and in the counties using the Becton Dickinson test capable of detecting the nvCT, the decrease was from 19% in 2007 to 5% in 2015. The rates of PID and ectopic pregnancy were higher in the counties using the test unable to detect the nvCT, but this effect was marginal at the national level in Sweden. The emergence of the nvCT seemed to be clonal, as all of the nvCT strains were of genotype E, and a subset of the nvCT strains analysed had identical *ompA* genes and was identical in an MLST analysis (Dahlberg et al. 2018). The nvCT did not appear to have an enhanced biological fitness *in vitro*, and the rapid spread in Sweden was probably due to the strong diagnostic selective advantage (Unemo et al. 2010). Indeed, a recent study based on a mathematical model proposed that the nvCT was 5% less transmissible than the wild type of *C. trachomatis*, and a 17% reduction in the duration of infection for the nvCT compared to the wild type of *C. trachomatis* was observed (Smid et al. 2019). Future investigations on the FI-nvCT, initially detected in early 2019, might reveal whether this variant has an altered fitness or a different outcome of infection compared to the wild type, or if the missed cases due to the variant had an effect on the rates of *C. trachomatis*-associated sequelae.

The genomic mutations observed with the Swedish nvCT and the FI-nvCT are a natural consequence of the microbial evolution combined with the high diagnostic selective pressure on *C. trachomatis*. This might be seen as an alternative strategy for *C. trachomatis* to spread, as antibiotic resistance among *C. trachomatis* strains has remained rare. Detecting new variants of *C. trachomatis* represents a challenge for laboratories as these bacteria are being detected mostly by NAATs. Laboratories should monitor the incidence and positivity rates of *C. trachomatis* and participate in external quality assessment programmes. The use of dual-target NAATs and back-up NAATs for *C. trachomatis* should be considered in laboratories. At a national level, it could be of benefit if different NAATs are used in detecting *C. trachomatis* infections. If there is suspicion of failure of detection, specimens can be sent to another laboratory for confirmatory testing with another NAAT. In addition to the actions performed by the laboratories, the European Commission has regulations for the medical devices and *in vitro* diagnostic medical devices, requiring the manufacturers to perform post-market monitoring of the diagnostic tests. This could prevent the variants from spreading unnoticed.

6.4 PREVALENCE OF HSV, HHV-6, HPV AND *M. GENITALIUM* (IV)

Among the urogenital samples of young women participating in a *C. trachomatis* screening trial in study IV, HPV DNA was significantly more frequently detected among the *C. trachomatis* positives than the negatives (66% vs. 25%). This suggests greater sexual activity and exposure to sexually transmissible microbes among these individuals. Similarly, in an Italian study, HPV co-infection was frequent (58%) among *C. trachomatis* infected women and the prevalence of HPV was even higher (68%) among ≤25-year-old women (Seraceni et al. 2016). This is of importance, as *C. trachomatis* might be a co-factor in the development of cervical cancer (Castellsagué et al. 2014; Lehtinen et al. 2011). Globally, there is a relationship between HPV prevalence and age, as the highest rates are observed in women younger than 25 years with a decline in older age groups (Forman et al. 2012). The prevalence of HPV among young women is over 20% in Europe and North America (Forman et al. 2012).

The prevalence of HSV, HHV-6 and *M. genitalium* was not significantly different among the *C. trachomatis* positive and negative young women. HSV DNA was rarely detected (1%) in the urogenital samples of young women. This finding is in agreement with the previous Finnish data, as a prevalence of 3% of clinically diagnosed HSV cases was reported in women at twelve outpatient clinics in 1995–2006 (Hiltunen-Back et al. 2009), and approximately 2% of the clients at an STI

clinic were HSV culture positive in 1994–2012 (Kortekangas-Savolainen et al. 2014; Kortekangas-Savolainen & Vuorinen 2007). The seroprevalence for HSV-1 was 45% and for HSV-2 11% among pregnant Finnish women in 2012 (Puhakka et al. 2016). The global prevalence estimate for genital HSV infections was 0.8% (15–19 year olds) and 2% (20–24 year olds) for HSV1, and 4.6% (15–19 year olds) and 7.8% (20–24 year olds) for HSV2 (Looker, Magaret, May, et al. 2015; Looker, Magaret, Turner, et al. 2015). HHV-6 DNA was detected in 12% of the samples analysed. Similarly, an HHV-6 prevalence of up to 10% was shown in the vaginal secretions of non-pregnant women in the USA (Leach et al. 1994), whereas a prevalence of up to 26% was reported in the genital tract specimens of pregnant Japanese women (Maeda et al. 1997). HHV-6 DNA positivity among the women can be explained by reactivation or (re)infection of HHV-6, as this virus induces a lifelong latent infection in humans.

The global estimate for the prevalence of *M. genitalium* was 1.7% in under 25-year-old women (Baumann et al. 2018), which is slightly lower than the global prevalence of 3.8% for *C. trachomatis* in 15–49-year-old-women (Rowley et al. 2019). The prevalence of *M. genitalium* DNA was 3.2% among samples of young Finnish women participating in a *C. trachomatis* screening trial. Earlier, a somewhat higher prevalence of 5.6% for *M. genitalium* was reported among clients at an STI clinic in Finland (Hokynar et al. 2018). In the other Nordic countries, the prevalence of *M. genitalium* was 9.8% in Sweden, 4.9% in Norway and 9.0% in Denmark (Unemo et al. 2018). In Norway, the prevalence of *M. genitalium* is suggested to be lower due to enhanced testing, treatment, and partner notification. The samples in study IV were collected from women volunteering in an HPV vaccination trial and simultaneously in a *C. trachomatis* screening trial, and the sexual activity among the participants may have been higher. Due to this, the true population-based prevalence of HPV, HSV, HHV-6 and *M. genitalium* and the prevalence of *C. trachomatis* and HPV co-infection might be lower than the ones reported here.

In study IV, we detected two samples with a macrolide resistant *M. genitalium* among the ten samples positive for *M. genitalium*. Previously, mutations leading to macrolide resistance were detected in 31% of *M. genitalium* positive samples among clients at an STI clinic in Finland (Hokynar et al. 2018). In the other Nordic countries, the prevalence of *M. genitalium* macrolide resistance was 18% in Sweden, 56% in Norway and 57% in Denmark (Unemo et al. 2018). This reflects differences in the national treatment guidelines, as doxycycline is recommended in Sweden and azithromycin in Norway and Denmark in the treatment of *C. trachomatis* infections. Fluoroquinolone resistance in *M. genitalium* has been demonstrated to be extremely rare in Finland (Hokynar et al. 2018), hence it was not investigated here. *N. gonorrhoeae* was rarely detected in the samples collected from young women. Previously, a prevalence of 0.3% was reported for *N. gonorrhoeae* among

clients at an STI clinic in Finland (Hokynar et al. 2018). The global prevalence of *N. gonorrhoeae* was 0.9% in women 15–49 years of age in 2016 (Rowley et al. 2019). CRB were rarely (0.8%) detected among the urogenital samples.

The prevalence of HSV, HHV-6, HPV and *M. genitalium* did not significantly differ between those who cleared the *C. trachomatis* infection and those who did not. HHV-6 DNA was slightly more numerous among those who gave a *C. trachomatis* positive TOC sample than a negative TOC sample (19% vs. 10%), but this difference was not statistically significant. Earlier studies have reported interesting interactions between HHV-6 and *C. trachomatis*. HHV-6 induced persistence of *C. trachomatis* (Prusty et al. 2012) and *C. trachomatis* activated replication of latent HHV-6 (Prusty et al. 2013). Similarly, a study on another herpesvirus showed that a *C. trachomatis* and HSV co-infection drives *C. trachomatis* persistence *in vitro* (Deka et al. 2006). This might be of importance as persistent forms of *C. trachomatis* are more resistant to azithromycin (Wyrick & Knight 2004). As 13% of the TOC samples were positive for *C. trachomatis*, the effect of co-infections, especially those due to herpesviruses, warrants further studies.

The *ompA* genotype distribution of *C. trachomatis* was similar among those who cleared the infection and those who did not. The relation of genotype to the clearance of uncomplicated genital *C. trachomatis* has been investigated, but the results in these few studies have been inconclusive (Geisler 2010). This suggests that genotype may not have a major part in the resolution of infection. Multiple factors, including other biological characteristics of *C. trachomatis*, host immunity (Batteiger, Xu, et al. 2010) and infectious load (Walker et al. 2012) probably play a role in clearance.

Co-infections with *C. trachomatis*, *M. genitalium*, HSV, HHV-6 and HPV can have several interactions and consequences on the outcome of infection, but these interactions are only partially defined. The information on the local and global epidemiology of these sexually transmitted pathogens is important in infection control when infection management guidelines are developed. Study IV showed the high prevalence of a *C. trachomatis* and HPV co-infection among young women which could have pathological consequences in the cervix. In addition, study IV explored the prevalence rates of HSV and HHV-6 DNA in urogenital specimens among young women for the first time in Finland. This information has relevance, as previous studies have shown that both these pathogens can induce *C. trachomatis* persistence, which could hamper antibiotic treatment of infections. The overall prevalence rates of HSV, HHV-6, HPV and *M. genitalium* were similar to those reported elsewhere.

6.5 *C. TRACHOMATIS* GROWTH DYNAMICS AND GENE EXPRESSION (III)

The expression of the *C. trachomatis ompA*, *cpaf*, *tarp* and *tox* genes was investigated *in vivo* in cervical swabs with real-time RT-PCR-based analyses in study III. mRNA of the *ompA*, *cpaf*, *tarp* and *tox* genes was infrequently detected in cervical swabs. The five analysed *C. trachomatis* low-passage-number clinical isolates expressed these genes in *in vitro* infections. This suggests that the differences are likely to be due to the small amount of chlamydial mRNA in the cervical swabs and not due to the differences between isolates. This was also supported by serology, as antibodies against the proteins encoded by genes *ompA*, *cpaf*, *tarp* and *tox* were detected in sera from the *C. trachomatis* positive patients (our unpublished observation). The few earlier studies on the *in vivo* gene expression of *C. trachomatis* analysed cytobrush cervical samples (Jha et al. 2009) or synovial biopsies (Gerard et al. 2013) which probably contain more *C. trachomatis* cells than swab specimens. The use of cervical biopsies may have resulted in a larger number of positive samples in the real-time PCR assays, but this invasive sample type is clearly much more difficult to obtain.

The growth kinetics of the *C. trachomatis* reference strains and clinical isolates were analysed with the *ompA* PCR. All clinical isolates except one (E127) exhibited a slower growth rate than the reference strains. The reference strains and the clinical isolate E127 showed a doubling time of approximately two to three hours and the rest of the clinical isolates a doubling time of approximately four hours. Similarly, a generation or doubling time of two to three hours has been estimated for urogenital *C. trachomatis* reference strains (Borges et al. 2010; Miyairi et al. 2006). In addition, the reference strains in study III were more efficient in producing progeny than the clinical isolates. *C. trachomatis* reference strains are most probably adapted to HeLa229 cells and the *in vitro* growth conditions in contrast to the low-passage-number isolates cultured from clinical samples, which has been shown before (Borges et al. 2015; Nunes et al. 2007). In this study, the growth properties of the low-passage-number clinical isolate E127 resembled those of the reference strains. This isolate was cultured from a cervical sample of an asymptomatic *C. trachomatis* seronegative woman, whose partner was positive for *C. trachomatis*. Based on this clinical data available, she may have been experiencing her first *C. trachomatis* infection, but the ultimate factors explaining the faster growth rate of this clinical isolate remain undefined. The other clinical isolates analysed were from *C. trachomatis* seropositive women possibly having repeated *C. trachomatis* infections.

In study III, the *in vitro* expression of the *cpaf*, *tarp* and *tox* genes using five clinical low-passage-number isolates and reference strains type E and F was analysed with real-time RT-PCR-based analyses in HeLa229 cells for the first time at different

stages of the chlamydial developmental cycle. Dissimilarities were observed in the *C. trachomatis* gene expression profile between the clinical isolates and the reference strains. The expression of *cpaf* was high in the reference strains compared to the clinical isolates in the mid-phase (12–24 hpi) of the developmental cycle. The relevance of this is not known, as the previously identified target molecules and effects of CPAF during a chlamydial infection have been questioned (Chen et al. 2012). The expression of *tox* occurred earlier (at 12 hpi vs. at 24 hpi) in the reference strains and the clinical isolate E127 than in the other clinical isolates. Chlamydial cytotoxin has a role early in the entry phase of the developmental cycle (Belland et al. 2001), so this might also be associated with the faster growth of these strains. Only the expression of *tarp* was fairly similar in the clinical isolates and the reference strains. In study III, the peak of expression for *tarp* was observed later than for the other genes in the clinical isolates and the reference strains. Similarly, in earlier studies, *tarp* expression was categorised as late (24–36 hpi) for type L2 (Nicholson et al. 2003), or *tarp* was expressed at 8–40 hpi in type D (Belland et al. 2003).

The expression of *ompA* in the clinical isolates and reference strains peaked in the middle of the chlamydial developmental cycle at 24 hpi. This correlates with the need for MOMP as an important structural component of the outer membrane in dividing RBs forming new organisms (Caldwell et al. 1981). The expression of *ompA* occurred earlier in the reference strains and in the clinical isolate E127 than in the other clinical isolates. The expression of *ompA* in this study was similar to that in the previous studies (Belland et al. 2003; Gomes et al. 2005; Nunes et al. 2007), which suggests that the infection model used in study III is comparable to those used before. The dissimilarities in gene expression profiles between different clinical isolates are probably due to the intrinsic properties of the single isolates, because the growth conditions were identical. Some of the comparisons of gene expression have been made to previous studies utilising microarray or RNA sequencing techniques. However, it has been shown that the RT-PCR data correlate well with the microarray and RNA sequencing data (Belland et al. 2003; Ferreira et al. 2017; Nicholson et al. 2003).

Studying *C. trachomatis* gene expression and growth kinetics during *in vitro* and *in vivo* infections is essential in understanding disease pathogenesis and evaluating the effects that immune mediators or antimicrobials have on the *C. trachomatis* infection. Study III showed that the selection of the *C. trachomatis* strains for gene expression experiments can have an impact on the results, as clinical isolates acted differently compared to reference strains. The use of carefully characterised low-passage-number *C. trachomatis* isolates instead of reference strains in gene expression studies might better reflect the situation *in vivo*.

7. CONCLUSIONS AND FUTURE PROSPECTS

The intracellular bacterium, *C. trachomatis*, is a master in playing hide and seek. This was demonstrated with the re-emergence of an old disease, lymphogranuloma venereum, with a new often asymptomatic manifestation of the infection. In addition, the accumulation of deletions and mutations in the bacterial genome, including the target sequences of diagnostic NAATs leading to failing detection, has been witnessed twice with *C. trachomatis*. Causing asymptomatic infections and evading detection seem to be a survival strategy of *C. trachomatis*, enabling it to continue spreading unnoticed.

Typing of *C. trachomatis* remains important in epidemiological surveillance, revealing transmission networks and distinguishing reinfection from treatment failure. In the future, high-resolution genotyping techniques such as MLST and WGS will prove useful, especially in situations where a single genotype dominates in a population. Distinguishing LGV types from non-LGV types is important in the proper management of rectal infections, as LGV infections require a longer antibiotic treatment regimen compared to the treatment of urogenital infections. This thesis showed, for the first time since the 1930s, that LGV infections also occur in Finland among MSM, a group vulnerable to multiple sexually transmitted infections. Detection of LGV is still based on in-house PCR tests which are not available in all parts of the world leading to undiagnosed LGV infections. In addition to LGV-specific tests, commercial screening tests validated for the analysis of extragenital samples are needed, as *C. trachomatis* rectal infections are prevalent also among women in Finland and elsewhere. The evolution of *C. trachomatis* genomes seems to be an ongoing process and can seriously affect the laboratory diagnosis of infections. In this thesis however, the Swedish variant of *C. trachomatis* was rarely detected in Finland. Clinical laboratories should be aware of the ability of *C. trachomatis* to develop mutations which can lead to failing detection of infections.

The interactions and consequences of *C. trachomatis* co-infections with other sexually transmitted pathogens are numerous, but remain an understudied area of research. It is important to understand the local and global prevalence of sexually transmitted microbes in the management of infections. This thesis showed that *C. trachomatis* co-infections with HPV are extremely prevalent among young women in Finland. This warrants further studies as the co-infection of these agents is a pre-requisite for cervical cancer. This thesis revealed dissimilarities in the transcriptional gene expression patterns between low-passage-number clinical *C. trachomatis* isolates and reference strains, which suggests that the use of clinical isolates might better reflect the situation during human infection. In the future,

C. trachomatis gene expression studies will most probably be focused on the analysis of whole transcriptomes. In addition, the analysis of the *in vivo* gene expression of *C. trachomatis* will benefit from the development of more sensitive transcriptome analysis methods, such as single-cell RNA sequencing.

ACKNOWLEDGEMENTS

This thesis work was carried out at the Department of Virology, Faculty of Medicine, University of Helsinki. The head of the department, Professor Kalle Saksela, is acknowledged for providing excellent facilities to conduct this research work.

I owe my deepest gratitude to the supervisor of my thesis, Docent Mirja Puolakkainen. I truly appreciate her patient guidance during these years, even when I was on maternity leave twice and started to work at HUSLAB during this thesis project. Her vast knowledge, enthusiasm and ideas in *Chlamydia* research were truly infective. The valuable comments made by the reviewers of my thesis, Docent Minna Mäki and Docent Janne Aittoniemi, are highly appreciated and they really improved the contents of this thesis. I am highly grateful to Docent Taru Meri for accepting the invitation to become my opponent. My thesis committee members, Docent Raisa Loginov and Docent Leena Maunula, are warmly thanked for their support and the useful advice I received during this process. I am thankful to Heini Torkko for the thorough revision of the language of this thesis. All the co-authors of the original publications, Docent Eija Hiltunen-Back, Tiina Eriksson, Kari Natunen, Professor emeritus Jorma Paavonen and Professor Matti Lehtinen, are acknowledged for their valuable contribution, support and expertise in the field.

I warmly thank the present and former members of the *Chlamydia* research group, especially Eveliina Markkula, Anu Haveri, Outi Rautio, Laura Mannonen, Kati Hokynar and Anu Kaitonen. Eveliina, Anu H. and Outi were the ones who initially introduced me to lab work on *Chlamydia*. I want to thank Kati for her valuable collaboration and good company in the lab and during congress trips. Anu K. is acknowledged for her skillful technical assistance. All the people at the Department of Virology are warmly thanked for creating a friendly and relaxed atmosphere, which provided a safe “inclusion” for us chlamydiologists.

I wish to express my gratitude to the present and former heads of the Department of Bacteriology, HUSLAB, Anu Pätäri-Sampo and Docent Pentti Kuusela, for their positive attitude towards research work and for granting me leave of absence for thesis work. The director of the Division of Clinical Microbiology, HUSLAB, Docent Maija Lappalainen, is also acknowledged for supporting my thesis work. I am grateful to the friendly and helpful staff at the Department of Virology and Immunology, HUSLAB, and the Clinic of Venereal Diseases, Skin and Allergy Hospital, Helsinki University Hospital, for collecting the samples analysed in these studies. My lovely co-workers at HUSLAB, you are too many to mention, but I have really enjoyed your company in and outside the lab. Especially Tanja Holma and Reetta Sihvonen are thanked for many years of friendship.

The members of Frendit ry., Suvi, Anu, Sari, Helmi, Hanna and Taina, are thanked for all the fun times spent together, our summer cottage trips, and especially the traditional pre-Christmas parties. It is comforting to know that we can truly rely on each other for support, whatever life throws in our way.

I want to thank my mother, and my sister Anu and her family, for loving support. My parents-in-law, Matti and Sirkka, are also warmly thanked for always being there to help whenever needed.

This thesis would not have been completed without my dear husband Jarkko, who was holding down the fort and taking care of our beloved children, Kaisla and Aarno, while I was writing this thesis. I am truly grateful for his love and support.

The Academy of Finland, the Hospital District of Helsinki and Uusimaa, the Finnish Society against STIs and the Finnish Society for Study of Infectious Diseases, are acknowledged for the financial support of this thesis work.

Helsinki, March 2020

Suvi Korhonen

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